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Low Plasma HDL Cholesterol is Associated with Greater Risk for Cardiovascular Disease in Subjects with Metabolic Syndrome

Hana Mohammed Al-Yousef, RD

A Thesis

Submitted in Partial fulfillment of the

Requirements for the degree of

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Approval Page

Master of Science Thesis

Low Plasma HDL Cholesterol is Associated with Greater Risk for Cardiovascular Disease in
Subjects with Metabolic Syndrome

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List of Abbreviations

| | |
|--|-------|
| Action for Health in Diabetes | AHEAD |
| ATP-Binding Cassette Transporter A1 | ABCA1 |
| Alanine Aminotransferase | ALT |
| Aspartate Aminotransferase | AST |
| Body Mass Index | BMI |
| Blood Pressure | BP |
| Coronary Artery Disease | CAD |
| Cholesterol Ester | CE |
| Cholesterol Ester Transfer Protein | CETP |
| Coronary Heart Disease | CHD |
| Carbohydrates | CHO |
| Chylomicrons | CM |
| Carbohydrate Restricted Diet | CRD |
| C-Reactive Protein | CRP |
| Cardiovascular Disease | CVD |
| Ethylenediaminetetraacetic Acid | EDTA |
| European Group for the Study of Insulin Resistance | EGIR |
| Glycemic Index | GI |
| Glutathione Peroxidase | GPx |

| | |
|--|--------------|
| Glycosylated Hemoglobin | HbA1c |
| High Density Lipoprotein- Cholesterol | HDL-c |
| Intermediate Density Lipoprotein | IDL |
| Interleukin 1- β | IL-1 β |
| Interleukin-6 | IL-6 |
| Interleukin-8 | IL-8 |
| Insulin Resistance | IR |
| Lecithin Cholesterol Acyltransferase | LCAT |
| Low Density Lipoprotein | LDL |
| Low Density Lipoprotein- Cholesterol | LDL-c |
| Lipoprotein a | Lp (a) |
| Lipoprotein Lipase | LPL |
| Monocyte Chemoattractant Protein-1 | MCP-1 |
| Mediterranean Diet | MedD |
| Metabolic Syndrome | MetS |
| Monounsaturated Fatty Acids | MUFA |
| The National Cholesterol Education Program-Third Adult Treatment Panel | NCEP:ATP III |
| Nutrition Data Systems for Research Software | NDSR |
| The National Health and Nutrition Examination Survey | NHANES |
| Nuclear Magnetic Resonance | NMR |
| Nitric Oxide | NO |
| Oxidized Low-Density Lipoprotein | Ox-LDL |

| | |
|---|---------------|
| Phospholipids | PL |
| Paraoxonase-1 | PON-1 |
| Reverse Cholesterol Transport | RCT |
| Serum Amyloid A | SAA |
| Superoxide Dismutase | SOD |
| Scavenger Receptor B1 | SR-B1 |
| Type II Diabetes Mellitus | T2D |
| Total Antioxidant Capacity | TAC |
| Thiobarbituric Acid Reactive Substances | TBARS |
| Total Cholesterol | TC |
| Triglycerides | TG |
| Tumor Necrosis Factor α | TNF- α |
| Very Low-Density Lipoprotein | VLDL |
| Waist Circumference | WC |
| World Health Organization | WHO |
| Waist-to-Hip ration | WHR |

Abstract

Low plasma HDL cholesterol (HDL-c) has been recognized as a biomarker of cardiovascular disease (CVD) and diabetes. Also, low HDL-c is one of the characteristics of metabolic syndrome (MetS).

Objective: To assess whether low HDL-c and/or low HDL functionality are associated with higher risk for CVD in subjects classified with MetS.

Methods: Forty-subjects with MetS (11 men /29 women, 52.4 ± 9.5 years) participated in the study. Anthropometric data [weight, height, BMI, waist circumference (WC), blood pressure (BP)], fasting plasma biomarkers [lipids, glucose, liver enzymes: alanine aminotransferase (ALT), and aspartate aminotransferase (AST), plasma insulin, and glycosylated hemoglobin (HbA1c)], biomarkers of oxidative stress and inflammation, and biomarkers of antioxidants status [glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase, and total antioxidant capacity (TAC)] were measured. Lipoproteins particles were measured to identify the size and number. Also, the functionality of HDL was further assessed by measuring plasma apolipoproteins, paraoxonase-1(PON-1) and serum amyloid A1 (SAA1).

Results: Participants had a mean BMI of 32.3 ± 2.7 kg/m² ($29.8 - 39.3$ kg/m²) placing the majority in the obesity category. In terms of MetS characteristics, all subjects (100%) fit the criteria for WC; 63% either had high systolic BP, high diastolic BP, or both; 70% were hyperglycemic; 48% had elevated plasma triglycerides (TG), and 43% had low HDL-c. We divided the subjects into two categories, Low HDL-c (men < 40 mg/dL and women < 50 mg/dL) (n = 17) and Normal HDL-c (men \geq 40 mg/dL and women \geq 50 mg/dL) (n = 23). Those with Low HDL-c had higher

systolic BP (129.9 ± 9.7 vs 121.2 ± 14.2 mm Hg, $p < 0.05$), higher TG (166.6 ± 8.0 vs 115.5 ± 56.8 mg/dl, $p < 0.05$), and lower TAC (1.23 ± 0.9 vs 2.09 ± 1.5 mM Trolox equivalents, $p < 0.05$). In addition, strong negative correlations were found between HDL-c concentrations and each of the following parameters: WC ($r = -0.418$, $p < 0.01$), insulin ($r = -0.413$, $p < 0.05$) and ALT ($r = -0.324$, $p < 0.05$). Lipoprotein data showed that large VLDL, medium VLDL, and small LDL particles were significantly higher in the Low HDL-c group (9.4 ± 6 vs 5.6 ± 4.4 nmol/L, $p < 0.05$), (25.1 ± 11.3 vs 15.8 ± 8.2 nmol/L, $p < 0.01$), (822.5 ± 283 vs 522.4 ± 288.2 nmol/L, $p < 0.01$), respectively. Both large and total HDL particles were higher in the Normal HDL-c group (8.2 ± 4.1 vs 4.3 ± 1.9 μ mol/L, $p < 0.01$), (37.5 ± 4.4 vs 30.8 ± 4.8 μ mol/L, $p < 0.01$), respectively. Apo A-I was significantly higher in Normal HDL-c group (985.4 ± 274.2 vs 790.7 ± 188.1 mg/L, $p < 0.05$). Also, Apo A-I was positively correlated with total HDL ($r = 0.671$, $p < 0.01$) and large HDL particles ($r = 0.530$, $p < 0.01$), respectively. PON-1 activity was significantly higher in the Normal HDL-c group (26.7 ± 14.1 vs 12.4 ± 16.1 U/ml, $p < 0.05$).

Conclusion: These data suggest that in these men and women with MetS, measuring both HDL-c concentrations and HDL particles can provide important information about levels and functionality of the protective HDL. In regards to plasma HDL-c, subjects with Low HDL-c appeared to have higher risk for CVD including dyslipidemia, hypertension, and higher concentrations for more atherogenic lipoproteins. Further, TAC, Apo A-I and PON-1 activity were lower among this group. These results confirm that individuals with Low HDL-c and MetS have increased risk for CVD.

Chapter 1

Introduction

Cardiovascular disease (CVD) is estimated to be the major cause of death with an estimate of 31% deaths per year globally.¹ CVD is a health concern that can occur by different modifiable risk factors including tobacco smoking, dyslipidemia, hypertension, and obesity. When there is an intake of excess energy coupled with physical inactivity, an epidemic of obesity increases affecting optimal health. Another health issue correlated with obesity that increases the risk factors of CVD is metabolic syndrome (MetS). In the United States, it has been estimated that nearly 35% of adults have MetS². This metabolic abnormality is defined by having three or more of these clinical parameters; central adiposity, high blood pressure, hyperglycemia, high plasma triglycerides (TG), and low HDL-cholesterol (HDL-c) concentrations³. Another factor that can contribute to an increase of CVD risk is the high concentrations of LDL-cholesterol (LDL-c)⁴. These abnormalities can further increase an individual's risk for both CVD and type II diabetes mellitus⁵. Plasma low HDL-c as one of MetS criteria has been shown to have an inverse relationship with the risk for coronary heart disease (CHD), even at low LDL-c levels, below 70 mg/dL⁶. Observational studies have been estimated that for each increment of 1 mg/dL in HDL-c, there is an approximate 2–3% reduction in CVD risk^{7,8}. This relationship is thought to be due to the cardioprotective properties of HDL. The most known function of HDL is that it mediates efflux of cholesterol ester (CE) from peripheral cells and transfers the excess CE to the liver for excretion⁹, in a process called reverse cholesterol transport (RCT). Other cardioprotective properties of HDL are: 1) inhibition of the expression of endothelial adhesion molecules and its action as anti-inflammatory agent; 2) antioxidative effects due to the association of HDL with several antioxidant

enzymes such as paraoxonase-1 (PON-1), glutathione peroxidase (GPx), and superoxide dismutase (SOD); 3) prevention of oxidized LDL (Ox-LDL) induced apoptosis; and 4) regulation of platelet adhesion^{10,11}. The HDL particle has two main key properties, the first one is its ability to bind to receptors on both hepatic and non-hepatic cells such as; ATP-binding cassette transporter A1 (ABCA1) and scavenger receptor B1 (SR-B1)^{12,13}. Another key property is enhancing the responsible enzyme for esterification of free cholesterol to CE through apoA-1 components, which is known as plasma lecithin-cholesterol acyltransferase (LCAT) activity¹⁴. HDL particles are highly heterogeneous in size, shape, density, cholesterol, phospholipids as well as apolipoproteins content^{11,15,16}. Usually, in clinical settings, plasma HDL-c concentrations are measured as a predictor and an independent risk parameter for CVD¹⁶⁻¹⁸. In contrast, recent data suggest that even with high HDL-c concentrations, the incidence of atherosclerosis is not reduced¹⁷, and very large HDL particles may induce the cardioprotective properties of the functional HDL¹⁸. Interestingly, in two large trials in high-risk groups, it was shown that the occurrence of cardiac events was significantly and inversely associated with raised HDL particle numbers but not plasma HDL-c concentrations¹⁹, and high HDL particle numbers significantly reduced CHD death by around 50%²⁰. Therefore, measuring both the HDL-c concentrations as well as parameters for HDL functionality in high-risk populations such as individuals with MetS is of great of interest. The aim of the present study was to analyze whether HDL-c and/or HDL functionality are associated with additional biomarkers of CVD in subjects classified with MetS. For that, we hypothesized that subjects with MetS with low HDL-c will have a greater risk for CVD as determined by clinical biomarkers, than subjects with MetS and normal HDL-c.

Chapter 2

Review of Literature

2.1 Cardiovascular Disease

Worldwide, the prevalence of obesity nearly doubled between 1980 and 2008 with cardiovascular disease (CVD) remaining the second leading cause of death globally¹. As reported, obese individuals have 3.5 times higher probability of having 3 risk factors for CVD and 2.4 times higher possibility of elevated plasma cholesterol¹. There are an estimated 62 million individuals diagnosed with CVD in the U.S.^{21,22}. It was estimated that 75% of patients with heart failure had a history of hypertension²³. As reported, the exact cause of CVD is unknown but there are well-known factors that increase the risk of CVD²⁴. CVD risk factors can be categorized into two groups, nonmodifiable and modifiable risk factors. The nonmodifiable risk factors include those that we cannot control, such as age, gender, ethnicity, and family history. In contrast, the modifiable risk factors are those that we can control and manage, such as dietary intake, weight, abdominal obesity, smoking, high blood pressure (BP), and type 2 diabetes mellitus (T2D). Abdominal obesity is one of the CVD risk factors and also a biomarker of metabolic syndrome (MetS) that can be measured by calculating waist circumference (WC) and waist-to-hip ratio (WHR). The increase of visceral adipose tissue can promote insulin resistance, hypertension, and dyslipidemia^{25,26}. As reported, after adjusting for age, each 1 cm increase in WC and 0.01 increase in WHR is associated with an increase of 2% and 5% respectively, of CVD²⁷. In the Framingham Heart Study, 5,881 participants were followed up to evaluate the relationship between body mass index (BMI) and the incidence of heart failure²⁸. Heart failure developed in 496 participants within 14 years follow up, and data showed that the incidence of heart failure increased with the increase

of BMI in both genders²⁸. It was reported that for each increment increase of 1 unit in BMI, the risk of heart failure was increased in men and women about 5% and 7%, respectively²⁸. Hypertension is another risk factor of CVD and one of the MetS features. In the U.S., it was estimated that approximately 50 million individuals have hypertension²¹. The prevalence of hypertension and T2D was also increased with the increase of BMI²⁸. Low plasma high-density lipoprotein cholesterol (HDL-c) level is an important risk factor of CVD as well as a MetS biomarker. Studies have been shown that low HDL-c concentrations are associated with increased mortality^{29,30}. High levels of oxidized low-density lipoprotein (Ox-LDL) and lipoprotein a [Lp(a)] are abnormal lipoprotein profiles associated with CVD in MetS³¹. It has been observed that obese individuals who met MetS criteria have higher levels of oxidative stress and inflammatory markers including tumor necrosis factor- α (TNF- α), interleukin 8 (IL-8), and C-reactive protein (CRP) than those without MetS³².

2.2 Metabolic Syndrome

Metabolic syndrome is a condition with a combination of metabolic disorders that increases the risk of CVD. This condition leads to increasing the risk of heart disease by 2-fold and T2D by 5-fold⁵. The prevalence of this disorder is increasing, and it was estimated to be 25% worldwide³³. Different factors can contribute to the development of MetS such as excess body weight, physical inactivity, rich fat and carbohydrate diets that can contribute to central obesity and insulin resistance (IR)³³. Young individuals with MetS can suffer from serious health complications in their later years, thus controlling this condition and reducing its prevalence should be the main goal. Weight loss, exercise and healthy diet seem to have the biggest effect on health and help to reduce MetS parameters. A common feature for MetS subjects is the excess body fat (especially visceral fat), studies have been shown that fat accumulated in the visceral area was associated with

hypertension, T2D, and atherogenic dyslipidemia^{26,34}. Atherogenic dyslipidemia is characterized by increased triglycerides (TG) and low HDL-c. These abnormalities can accelerate the risk of CVD, especially in a high-risk group as MetS.

2.2.1 Definition

In history, the term (metabolic syndrome) is a debated topic. In 1975, a German researcher, Hermann Haller coined this term³⁵. Also, in 1988, Gerald M Reaven described a phenomenon that occurs in individuals with coronary artery disease (CAD) that includes a combination of the following; IR, hyperglycemia, hyperinsulinism, hypertriglyceridemia, hypertension, and low plasma concentration of HDL-c, as Syndrome X. After that the interest to identify this phenomenon increased³⁶. There were different criteria set out by different associations to define and diagnose this condition. The World Health Organization (WHO) was the first to define MetS criteria in 1988, as a presence of high insulin levels in addition to two of the following factors: abdominal obesity, hypertriglyceridemia and low HDL-c; hypertension, or microalbuminuria. In 1999, the European Group for the Study of Insulin Resistance (EGIR) suggested that the term *insulin resistance syndrome* was more appropriate and modified MetS criteria to have high fasting insulin concentrations plus two other factors out of four: abdominal obesity; high TG and low HDL-c; high BP; or high fasting plasma glucose levels³³. The cutoff of these factors was different than those used by WHO. Another association set a criterion of MetS and became the most common guidelines was the National Cholesterol Education Program –Third Adult Treatment Panel III (NCEP:ATP III) in 2001, and defined MetS as the having three of the five clinical measurements which include high WC, high BP, high fasting glucose, hypertriglyceridemia, and low HDL-c³.

2.2.2 Parameters of MetS

According to NCEP: ATP III guidelines, there are five metabolic disorders and the presence of three of these features, will meet the definition of MetS. Abdominal obesity has a greater impact on health and can increase CVD risk factors^{37,38}. Abdominal obesity as WC has cutoffs that are different based on gender. For males, WC must be equal to or greater than 102 cm (40 inches) to meet the guidelines. For females to meet WC criterion, it must be equal to or greater than 88 cm (35 inches). Dyslipidemia is characterized by higher TG and lower HDL-c concentrations. Greater than or equal to 150 mg/dL is considered to be high TG. The cutoff of HDL-c concentrations is also based on gender. Less than 40 mg/dL (1.0 mmol/L) for males is considered low HDL-c concentrations. For females, the cut-off point of HDL-c is less than 50 mg/dL (1.3 mmol/L). Another clinical measurement, common among obese individuals with IR is high BP. The guidelines defined that having the systolic BP equal to or over 130 mm Hg and the diastolic BP equal to or over 85 mm Hg is a criterion of MetS. Obesity itself causes and aggravates IR which is linked to several CVD risk factors, such as hyperglycemia and T2D³³. High fasting glucose or in another term hyperglycemia is common among obese individuals when insulin in the body cannot regulate glucose levels after a meal intake. Thus, this can lead to elevated plasma glucose remaining in circulation. The cutoff of fasting plasma glucose was modified in 2004 from greater than or equal to 110 mg/dL (6.1 mmol/L) to greater than or equal to 100 mg/dL (5.6 mmol/L), in accordance with the American Diabetes Association's updated standard of normal fasting glucose level³⁹.

2.2.3 Prevalence of Risk Factors

According to the National Health and Nutrition Examination Survey (NHANES) 2003–2006 and NCEP:ATP III guidelines, the most prevalent of MetS criteria were central obesity (53%), hypertension (40%), and hyperglycemia (39%), and the least prevalent were

hypertriglyceridemia (31%) and low HDL-c (25%)⁴⁰. There were differences in the prevalence of MetS risk factors by gender. As reported, males had a higher age-adjusted prevalence of hypertension, hyperglycemia, and hypertriglyceridemia whereas females had a higher age-adjusted prevalence of central obesity and low HDL-c concentrations⁴⁰. It seems that among females the prevalence of each risk factors was increased with age. For males, the prevalence of hypertension and hyperglycemia was increased with age, but for central obesity, hypertriglyceridemia, and HDL-c were no significant differences with age⁴⁰.

Based on race and ethnicity, the age-adjusted patterns varied between groups. Among males, non-Hispanic whites and Mexican-Americans had a higher prevalence of hypertriglyceridemia and low HDL-c than non-Hispanic blacks⁴⁰. Non-Hispanic white males had a higher prevalence of central obesity than non-Hispanic black males, but non-Hispanic blacks had a higher prevalence of hypertension than Mexican Americans⁴⁰. For females, central obesity and hyperglycemia were more common among non-Hispanic blacks and Mexican Americans than non-Hispanic whites⁴⁰. Non-Hispanic blacks had the highest prevalence of hypertension, while Mexican Americans had the highest prevalence of low HDL-c and hypertriglyceridemia⁴⁰.

When BMI increased, the prevalence of each of the five risk factors as well as the prevalence of MetS was increased for both genders⁴⁰. Overweight males were more than 6 times and obese males were more than nearly 32 times as likely to meet MetS criteria than males with normal weight or underweight⁴⁰. Among females, overweight was 5.5 times and obese was 17 times more likely to meet this criterion than normal and underweight⁴⁰. It was reported that between 2003–2004 and 2013–2014, there were no significant changes in childhood obesity prevalence, but among adults there was an increasing trend⁴¹.

As reported, males and females 40–59 years old were around three times more likely to meet MetS criteria than those of younger age⁴⁰. 25% of non-Hispanic black males met the ATPIII criteria compared to 37% of non-Hispanic whites⁴⁰. In contrast, both non-Hispanic black and Mexican American females were about 1.5 times more likely to meet MetS criteria than non-Hispanic white⁴⁰.

2.3 Lipoproteins Overview

Lipoproteins are clusters of lipids associated with proteins and serve as transport vehicles for lipids in the lymph and blood. Typical lipoproteins consist of hydrophobic molecules with TG and cholesterol on the inside surrounded by hydrophilic phospholipids (PL) on the outside. The plasma lipoproteins have major classes defined by density and size at which they are isolated. Chylomicron (CM), very low-density lipoprotein (VLDL), and there is the intermediate density lipoprotein (IDL), which is the transit form of VLDL conversion to low density lipoprotein (LDL), and there is HDL⁴².

After dietary fat intake, CM are produced and secreted by the enterocytes into the lymph, and they require apo B-48. These chylomicrons are TG-rich particles with around 82% TG. In addition, CM have, cholesteryl esters (CE), PL, vitamins A and E in the core and a monolayer of PL, free cholesterol, and protein on the surface⁴³. CM also have apo E, apo C-II and apo C-III that were acquired from HDL in circulation. After the action of the enzyme lipoprotein lipase (LPL), the CM particles become smaller and returns to the liver as chylomicron remnant⁴³. Typically, CM density is 0.92 - 0.96 g/ml with diameter approximately 100-500 nm⁴².

VLDL particles are made primarily in the liver and they require apo B-100 for secretion. Around half is TG, and they also have apo A-I, apo A-II, apo E, apo C-I, apo C-II, and apo C-III⁴³. VLDL particle density is 0.95- 1.006 g/ml and about 30-80 nm in diameter⁴². During VLDL

circulation, TG is donated to extra-hepatic tissues by the action of LPL⁴³. As the TG is removed from VLDL, IDL is formed and then converted to LDL⁴³. IDL particle density is heavier than VLDL with about 1.019 g/ml and 25-50 nm in diameter⁴². High concentrations of plasma IDL are associated with increased risk of CVD⁴⁴.

LDL is known as a cholesterol rich lipoprotein that contains only apo-B100, and can be removed by LDL receptor on the liver and extra-hepatic tissues⁴³. LDL particle density is about 1.029-1.063 g/ml and has different subclasses⁴². Elevated plasma LDL-c is a risk factor for the development of CVD³¹. LDL-c is positively correlated with CAD and CVD mortality in the presence of MetS condition²⁰. The heaviest lipoprotein and the smallest one is HDL, which will be discussed below in more detail.

2.3.1 HDL Composition and RCT

As mentioned above, HDL has the highest density with 1.063- 1.210 g/ml, and the smallest size (15-20 nm). HDL can originate from both the liver and intestine as a nascent particle devoid of lipids but containing apo A-I. Discoidal HDL particles interact with ATP-binding cassette transporter A1 (ABCA1), which is located at peripheral tissues, then free cholesterol will be esterified by the action of the enzyme lecithin cholesterol acyltransferase (LCAT) to form the spherical α -HDL. LCAT is activated by the major apolipoprotein in HDL, apo A-I¹³. Half of each HDL particle is composed of PL, unesterified cholesterol, TG and exchangeable apolipoproteins such as, apo A-I, apo A-II, apo E, apo C-I, apo C-II, and apo C-III⁴³. The other half consists of proteins, and according to the proteomic studies, they discovered that more than 100 proteins are in HDL particles in addition to the apo As⁴⁵. HDL particles play a critical role in transporting the excess cholesterol from the peripheral tissues to the liver for elimination⁴⁶. This beneficial process is known as reverse cholesterol transport (RCT). RCT is inversely correlated with CVD in that it

plays an important role in modifying the formation and development of fatty plaques⁴⁷. There are different HDL subclasses, nascent HDL, HDL₂ (larger and less dense), and HDL₃ (smaller and denser). Nascent HDL known as pre- β HDL has low lipid content while α -HDL (HDL₂+HDL₃) has high lipid content⁴⁸.

RCT occur in three stages; extravascular, intravascular, and intrahepatic⁴⁶. The first stage is cholesterol efflux from cell membranes and it is transported between cell membranes via the action of ABCA1, which is located on extra-hepatic tissues and generates structures that facilitate the interaction with lipoproteins. Thus, the HDL particles uptake the unesterified cholesterol as well as antioxidant substances such as, vitamin E, Lutein and Zeaxanthin via the action of apo A-I in HDL particles and ABCA-1 on the intestine⁹. The second stage is the esterification of free cholesterol in HDL by the action of the enzyme LCAT, and some of the formed CE is transferred to CM and VLDL by cholesteryl ester transfer protein (CETP) while some will remain in the HDL core to increase its size and decrease the density^{9,46}. HDL particles are taken up by the liver through two pathways. The direct pathway is through apo A-I, which is a ligand for hepatic scavenger receptor BI (SR-BI) where selective lipid uptake occurs, then the cholesterol is directed for biliary excretion. The indirect pathway is through hepatic LDL- receptor, and by the action of CETP, which facilitates the exchange of TG from the apo B containing lipoproteins (VLDL, LDL, CM) with CE from HDL^{9,43}. The RCT is finalized by the biliary excretion⁴⁶.

2.3.2 HDL Health Benefits

In addition to removing excess plaque lipids through the RCT process, HDL exhibits anti-inflammatory properties through interactions with the vascular endothelium and circulating inflammatory cells^{43,49}. HDL has antithrombotic and antioxidant properties and improves

endothelial function, as well as expression of inflammatory mediators⁴³. HDL particles have an important capacity to carry antioxidants⁴⁷. Abnormal HDL particles promote endothelial superoxide production and reduce nitric oxide (NO) bioavailability, thus, causing endothelial dysfunction, increasing BP and increasing cellular inflammation⁵⁰. In contrast, normal HDL particles have favorable effects on endothelial cell proliferation, and promote NO production⁴⁹.

Also, it has been shown that HDL has antiapoptotic functions for a number of cell types such as, smooth muscle cells, some leukocytes, pancreatic β cells, cardiomyocytes, and bone-forming cells^{43,49}. Recent studies in humans suggest that HDL-c was inversely associated with CVD^{30,50}. The Framingham Heart Study identified CVD risk factors such as low plasma HDL-c concentrations, which were associated with a higher mortality rate²⁹. It has been estimated that for each increment of 1 mg/dL in HDL-c, the CHD risk is reduced by 2% - 3% in men and women, respectively⁷. In a 10-year prospective study of male employees, it was reported that the development of ischemic heart disease was significantly higher among males with low HDL₂, and high levels of LDL, IDL, and small VLDL⁵¹.

Despite previously mentioned HDL benefits, HDL particles can be modified to become dysfunctional and pro-atherogenic particles under certain conditions such as, obesity, MetS, and chronic inflammation^{30,52,53}. HDL is considered an anti-atherogenic lipoprotein, but HDL levels in unhealthy individuals is sometimes less efficient. Obesity and MetS affect HDL particles and cholesterol, HDL become smaller in size and less dense, which will impair its anti-atherogenic and anti-inflammatory properties^{54,55}. All the three HDL subclasses are involved in RCT. However, there were negative correlations between HDL₂ (larger) and CVD^{31,46}. In addition, it has been suggested that HDL₃ particles (smaller) may have greater protective effects than the larger particles⁵⁶. This topic remains controversial.

2.3.3 HDL particles and HDL-c Measurements

It is well known that plasma HDL-c concentrations are negatively associated with atherosclerotic CVD, especially CAD. HDL-c can be measured in plasma and serum. Previous clinical trials that aimed to raise HDL-c by pharmacology drugs including fibrates, niacin, and CETP inhibitors have failed to show health benefits or to improve CVD biomarkers and reduce CVD mortality^{57,58}. However, previous studies indicate that HDL particles concentrations can be superior to HDL-c concentrations as a predictor of CVD⁵⁹. Measurements of HDL particle number and size become an improved assessment of cardiovascular risk⁶. HDL constitutes a heterogeneous group of particles that differ in density, size, lipid composition, and apolipoprotein content, thus HDL can be fractionated into discrete subclasses by different techniques¹². HDL separation can be done by charge as capillary isotachopheresis⁶⁰, and HDL size by using one of the following: gel gradient electrophoresis separation⁶¹, Fast liquid chromatography, Ion exchange chromatography⁶², or Nuclear magnetic resonance (NMR) by proton spectroscopy. Gradient gel electrophoresis can separate HDL into two fractions on the basis of density, HDL₂ (1.063-1.125 g/ml) and HDL₃(1.125-1.21 g/ml)¹². These particles can be further divided into sub-fractions, HDL₂ into HDL_{2b} (10.6 nm) and HDL_{2a} (9.2 nm), and HDL₃ into HDL_{3a} (8.4 nm), HDL_{3b} (8.0 nm), and HDL_{3c} (7.6 nm)¹². NMR is a technique that quantifies the number and size of VLDL, IDL, LDL, HDL particles and expresses each as lipoprotein particle concentration and as an average particle size⁶³. It quantifies more than 30 lipoprotein fractions that can be grouped into 10 subclasses based on diameter: large VLDL (> 60 nm), medium VLDL (35–60 nm), small VLDL (27–35 nm), IDL (23–27 nm), large LDL (21.2–23 nm), medium LDL (19.8–21.2 nm), small LDL (18–19.8 nm), large HDL (8.8–13 nm), medium HDL (8.2–8.8 nm) and small HDL (7.3–8.2 nm)⁶³.

Methods for measurement of HDL sub-fractions as mentioned before and measuring HDL functionality may also be more effective in predicting CVD risk than measuring plasma HDL-c concentrations alone. It is suggested that future research should determine the remodeling of HDL particles in unhealthy conditions⁵⁰. Obesity and MetS are conditions that alter HDL particles and reduce HDL-c concentrations⁶⁴. There is evidence that the small HDL particles mostly seen in MetS are functionally defective⁵⁶. Further, small HDL particles were observed along with CVD⁶³. In contrast, large HDL particles are strongly inversely associated with CVD⁶³. Among MetS, abnormalities of lipoproteins particles were observed⁶⁴. However, the relationship between sizes and numbers of HDL particles and the MetS parameters have not been fully addressed.

2.3.4 HDL Functionality

During tissue injury, inflammation status will induce the acute phase in which the liver increases the synthesis of acute-phase protein, which is serum amyloid A (SAA) and its isoforms, 1 and 2¹⁴. It is known that proinflammatory cytokines including interleukin 1- β (IL-1 β), interleukin 6 (IL-6) and TNF- α play important roles in hepatic expression of SAA response to acute-phase⁶⁵. Elevation of plasma SAA levels causes mainly HDL₃ remodeling, by which SAA displaces apo A-I to become an apolipoprotein of HDL and may alter HDL metabolism and cholesterol transport¹⁴. SAA may also promote a pro-inflammatory and pro-atherogenic HDL through limiting its ability to promote RCT⁶⁶. Another important marker that significantly associates with CHD and has impaired the antiatherogenic properties of HDL particles is apo C-III. These findings are based on an observational study, which analyzed HDL-apo C-III levels in patients with and without CHD⁶⁷. Patients with CHD were on statin treatment. After adjusting of covariates, among CHD patients with statin, plasma HDL along with elevated plasma apo C-III, and even with elevation of HDL-c concentrations, there was remaining CVD risk among CHD patients⁶⁷. Thus, SAA1 and

apo C-III are considered markers for dysfunctional HDL particles. In contrast, apo A-I, apo-E and Paraoxonase-1 (PON-1) are markers for functional HDL particles. Apo A-I is one of the major HDL apolipoproteins that is required for normal HDL biosynthesis. Higher levels of HDL-c have been shown to significantly reduce CVD risk and in particular higher levels of apo A-I were negatively associated with CVD risk¹⁶. PON-1 is an enzyme that is synthesized mainly in the liver and secreted into the blood where it associates with HDL particles⁶⁸. PON-1 has atheroprotective effects including inhibition of excess cholesterol synthesis and promotion of cholesterol efflux. PON-1 has also, anti-inflammatory functions and raises antioxidant effects of HDL⁶⁸. Paraoxonase-1, 2, and 3 have antiatherogenic properties including delayed pro-atherogenic oxidation of LDL and cell membranes to prevent atherosclerosis development⁶⁹. It was reported that due to lower PON-1 activity the interaction with SR-B1 receptors was less efficient among CHD patients when compared it with healthy individuals⁷⁰. Thus, HDL associated PON-1 protects against CVD through previously described mechanisms of the action of PON-1.

2.4 Therapeutics

The primary goal of caring for individuals with MetS is to reduce the clinical atherosclerosis disease risk factors. First-line therapy should focus on the major risk factors, such as elevated LDL-c, reduced functional HDL-c, hypertension, and prediabetes. Individuals with MetS rarely have normal body weight and are physically active. Therefore, lifestyle modifications including decreases in body weight, increases in physical activity, or following anti-atherogenic diets could be the key emphasis in MetS management. According to the American Heart Association, the first line of therapy should be a healthy diet and increased physical activity to achieve weight loss⁷¹. Studies have shown an improvement in serum cholesterol, TG, HDL-c, inflammatory markers, BP and IR⁷²⁻⁷⁴. In the last 50 years, studies have been focused on examining

the relationship between health and diet. Many studies assessed the role of different diets on health benefits and decreases in the risk for disease⁷⁵.

2.4.1 Energy-Restricted Diets

Energy-restricted diets are the most common dietary strategies for reducing excess weight and related comorbidities. There is a variety of options for planning and monitoring a reduced kilocalorie diet. While some strategies recommend energy reduction without restricted food types, others are using the exchange systems to plan energy and nutrient intake⁴². The exchange system that has the flexibility to choose the daily amounts of calories from an exchange list of foods was established by American Diabetes and American Dietetics Associations⁷⁶. The main approach of choosing a weight management plan is to be based on nutrition and exercise principles, suitable for each individual based on their preferences to promote long-term lifestyle changes to reduce weight effectively and to teach meal planning skills⁷⁶. The common problem in weight loss plans is helping the individual to maintain the weight loss long-term, rather than follow hypocaloric diets for a specific period of time, and face the difficulties to follow the new dietary habits. For individuals who are overweight or obese, which is the case for most individuals with MetS, weight loss is an urgent step as it is associated with the improvement of the individual metabolic profile of MetS. Weight loss is associated with improvement of visceral obesity, high blood pressure, and high fasting glucose⁷⁷.

Further, weight loss can also improve the low-grade inflammation condition among individuals with MetS⁷⁸. At the same time, body weight reduction is associated with the improvement in plasma glucose levels and IR. In studies done with diabetic overweight/obese subjects [The Look AHEAD (Action for Health in Diabetes), n= 5,145] have shown that 5-10% loss of body weight can improve participant's fitness level, reduce glycosylated hemoglobin

(HbA1c), and improve CVD risk factors, such as decreasing high BP, decreasing fasting glucose levels, increasing plasma HDL-c, decreasing LDL-c, and decreasing TG concentrations⁷⁹. Also, there was an evidence that even small weight reduction (5-10%) can reduce CVD risk factors, and the guidelines recommend a 5-10% loss of weight as an initial goal to obtain health benefits^{80,81}.

2.4.2 Moderate/Low Carbohydrate Diet

The Food and Nutrition Board, Institute of Medicine, and National Academies recommendations set an Acceptable Macronutrient Distribution Ranges for 18+ years old daily intake for both genders, the percentages range are 45–65, 10–35, 20–35 for carbohydrate (CHO), protein, and fat respectively. The amount vs quality of CHO intake is a controversial topic, some studies suggested that the CHO reduction has health benefits especially with great weight loss and improved lipid profile^{82–84}, while others suggested that the quality of CHO as measuring with glycemic index (GI) is more important as the quantity. GI is a method that consists of a ranking on a scale from 0 to 100 for classifying CHO-containing foods according to the postprandial glucose response⁴². The consumption of high GI-CHO has been associated with hyperglycemia, IR, T2D, and hypertriglyceridemia^{85,86}. These abnormalities are directly related to MetS.

Moderate, low CHO diets are different on their CHO percentages, for moderate the CHO distribution can be around 40 to 55% of daily energy requirement. In two studies done with T2D subjects, moderate CHO intake (40%) has shown health benefits^{83,87}. Low CHO intake can reach 5-30% energy from CHO. Consumption of low CHO diets has shown benefits to decrease plasma TG, decrease small LDL, decrease inflammatory markers, and increase HDL-c. In addition, it has a role in reducing blood pressure and increasing weight loss⁸⁸. When total fat is reduced, CHO intake is increased and healthy unsaturated fats are decreased⁸⁹. Although consumption of low-fat diets was associated with significant weight loss, CVD rates did not decrease⁸⁹. After comparing

these two diets, it seems that low CHO diet had further health benefits for increasing HDL-c significantly with a decrease in phenotype B of LDL, which is the small dense LDL particles that are considered more atherogenic with high susceptibility to become oxidized due to short lag phase and greater concentrations of conjugated dienes^{76,90}.

In one study, researchers examined the effect of approximately 10% CHO daily intake on weight loss and MetS parameters. Their participants checked their ketone levels through urine tests for maintaining ketones production after following the carbohydrate-restricted diet (CRD)⁹¹. In contrast, high CHO intake has been linked with an increase in MetS numbers regardless of fat intake in males and with higher MetS risk among females that consumed high CHO and low fat²⁴. For Asian subjects with MetS who had relatively low BMI and consumed low-fat diet and high CHO intake, the incidence of MetS and CVD was higher than in western countries⁹². These results may indicate that excess CHO intake, not fat intake is associated with MetS. In another study, subjects consumed 13% of carbohydrates for 12 weeks, and researchers observed a significant reduction in plasma lipoprotein (a) and a reduction in CRP and TNF- α , which are inflammatory markers⁹³. These findings are important because they add to the benefits of CRD in lowering inflammatory markers and plasma cholesterol. In subjects with MetS, a study was conducted to compare between consumption of CRD (20-25% CHO) and consumption of the American Heart Association diet (50-55% CHO)⁷³. The CRD group had large VLDL particles while medium and small LDL particles were decreased⁷³. In addition, apo B levels were significantly decreased⁷³. Many studies concluded that a reduced carbohydrate diet results in a spontaneous reduction in energy intake that may lead to weight and fat loss⁹⁴. Although CHO restricted diets are high in fat, the absolute amount of total fat is usually unchanged; and it has been shown that the only nutrient that is increased or maintained is protein^{24,95,96}.

2.4.3 Daily Egg Consumption

Egg consumption is one of the dietary strategies that has been associated with changes in small LDL and HDL subclasses to a larger one, and that has a positive effect on reducing CVD biomarkers⁶⁴. Participants with MetS have followed CRD along with consumption of 3 whole eggs/day for 12 weeks, large VLDL, small LDL-c, small IDL lipoproteins and insulin resistance were improved, and HDL numbers and sizes were increased⁶⁴. Large VLDL carries more TG, and smaller LDL and IDL are prone to be easily oxidized. Whereas larger HDL is favorable because of their roles in the RCT. Also, intake up to 3 eggs/day showed an improvement in HDL-c in healthy subjects⁹⁷. For individuals with MetS, intake of 3 eggs/day along with CRD had benefits on lipid profile and were safe to consume⁶⁴. Egg contents that might have role in influencing HDL-c concentrations are phosphatidylcholine and dietary cholesterol⁹⁸. Dietary phospholipid intake has been associated with the increase of HDL-c concentrations in human and animal studies⁹⁹. Egg consumption also had a role in RCT, in which the serum cholesterol efflux capacity was increased in subjects with MetS⁹⁸. Studies have shown that with egg intake, HDL particles and cholesterol levels were positively improved in healthy populations^{100,101} as well as in MetS⁶⁴. These findings indicate that eggs may be considered as a functional food that can promote beneficial shifts in HDL composition, metabolism, and function in both healthy and MetS populations.

Eggs contain carotenoids lutein and zeaxanthin, antioxidants that may protect against lipid oxidation¹⁰². In a study conducted in healthy populations, the consumption of 0, 1, 2, 3 eggs per day were evaluated¹⁰¹. Compared to no egg intake with the 1-3 eggs/day, large LDL and large HDL were increased¹⁰¹. Large particles of LDL are known to be the least atherogenic LDL

subfraction because they are less susceptible to modification and oxidation. Also, apo A-I and LCAT were increased with 1-3 eggs/day consumption while PON-1 activity was increased significantly with the intake of 3 eggs/day¹⁰¹.

2.4.4 Mediterranean Diet

In 1970, a researcher observed the lower incidence of CVD and certain types of cancer in some Mediterranean countries compared to the USA or Northern Europe and hypothesized that it was possibly due to the dietary habits of that population¹⁰³. The Mediterranean diet (MedD) is a diet consisting of fresh fish and seafood (as a source of omega-3), a moderate consumption of dairy products, poultry and eggs and a high consumption of plant-based foods, such as fruit, vegetables, legumes, nuts and seeds, and whole grains. Nut intake is mainly high relative to other diets. In addition, the intake of extra virgin olive oil, as the main source of dietary lipids and as a source of monounsaturated fatty acids (MUFA) is a unique characteristic for this diet. Another characteristic is low frequency and amounts of red and processed meat intake¹⁰⁴. Omega-3 and olive oil intake have been shown to protect against factors that increase CVD risk, such as its effect on weight, in a follow-up of a large Spanish cohort [The SUN project, Seguimiento University of Navarra], high intakes of olive oil were not associated with increased risk of subsequent weight gain¹⁰⁵. Also, adherence to the MedD has been shown to reduce BP, reduce dyslipidemia, increase insulin sensitivity, and reduce obesity (visceral obesity, which is a well-known CVD risk factor)^{74,75,106–108}. In addition, in the PREDIMED study, which was a multicenter, randomized, controlled, parallel-group clinical trial, that aims to test the efficacy of the traditional MedD as the primary prevention of coronary heart disease, a total of 800 subjects (55-80 years) at high CVD risk who followed the MedD with high consumption of nuts and extra virgin olive oil had reduced serum

concentrations of inflammatory markers¹⁰⁹. Since subjects with MetS have a low-grade inflammation, the MedD has been shown to have health benefits and can reduce MetS metabolic disorders, and the MedD is a lifestyle-friendly diet that could be an easy lifestyle modification for the overweight/obese group. The 2013 report guidelines on lifestyle management from the American Heart Association/ the American College of Cardiology⁷¹, and the European Society of Cardiology¹¹⁰ all recommend the MedD as a prevention to reduce CVD risk.

These types of diets can be the primary step to prevent and reduce CVD risk factors correlated with the presence of MetS. For that, lifestyle modification as dietary intervention and exercise are two important factors to reduce body weight and central obesity, which can be the key to prevent and solve this worldwide epidemic.

2.4.5 Exercise

Lifestyle modification is an important tool to reduce the future occurrence of MetS, these changes should include both dietary intervention and increased physical activity. Diet is a crucial key to weight loss and improve CVD risk factors as well as the activity level. According to NHANES 2011-2014, the prevalence of low HDL-c was significantly higher among adults who did not meet the recommended physical activity guidelines (21.0%) than adults who met the guidelines (17.7%)¹¹¹. In a meta-analysis about dietary intervention and/or physical activity on MetS subjects the long-term, moderate to moderately vigorous intensity exercise, three to four times per week, HDL-c concentrations were increased by 1.8 mg/dL along with decrease in TG levels by 19 mg/dL, those results were observed on 20 randomized control studies¹¹². Another meta-analysis about the effects of exercise on T2D subjects has been reported. Exercise types were either anaerobic, resistance or a combination of both, the inflammatory cytokines (CRP and IL-6) were decreased and leptin levels were improved significantly with aerobic exercise¹¹³. Studies

about the role of different types of exercise on subjects with MetS, such as aerobic exercise alone versus combination of different types of exercise are insufficient, and there is a need to investigate the role of different types of exercise on MetS parameters and whether they have different benefits on lipid profile, which has positive effects on MetS¹¹⁴. Also, adequate assessment of lifestyle modification is needed to clarify which aspects and what degree of lifestyle modification are best to resolve MetS.

In summary, increases in MetS parameters among individuals with excess body weight can increase risk for CVD and T2D. Both HDL-c and HDL particles can provide different benefits. HDL particles have atheroprotective properties including antioxidant and anti-inflammatory properties, improvements of endothelial function, and RCT process. HDL-c is a measurement that can give some information about HDL in the circulation. Low plasma HDL-c is one of the MetS markers and also an independent risk factor for CVD. Although pharmaceutical drugs such as niacin and statin have an effect to increase plasma HDL-c to higher levels, the prevalence of CVD and mortality did not change. Thus, measuring HDL functionality starts to gain attention in research. It is known that functional HDL particles are expected to increase cholesterol efflux, decrease inflammation, and decrease thrombosis. Some of the markers for functional HDL are apo A-I and PON1. On the other hand, dysfunctional HDL particles are expected to decrease cholesterol efflux, increase inflammation, and increase thrombosis. Both apo C-III and SAA1 are two markers for dysfunctional HDL. Both diet and exercise have an effect on plasma HDL-c concentrations and HDL particles. Therefore, controlling these two factors and measuring HDL functionality along with measuring plasma HDL-c could be important to understand the effects of lifestyle interventions.

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Chapter 3

Materials and Methods

3.1 Study Design

Data for this project was obtained from recruited subjects of a study conducted at the University of Connecticut¹. This observational study included men and women, aged 32-70 years old, and classified with MetS. The study was approved by the University of Connecticut Institutional Review Board (IRB H14-278). All subjects signed a written consent form prior to joining the study. The study was registered at Clinicaltrials.gov, protocol # NCT02531334.

Every subject that was accepted in the study underwent a screening evaluation to determine whether they classified for MetS. The screening involved anthropometric measurements including weight, height, waist circumference and BMI as well as plasma values for triglycerides (TG), HDL-c and glucose, and blood pressure (BP).

3.2 Subjects

Data were collected from 40 overweight and obese men (n=11) and women (n=29), 32-70 years old who met the proper criteria. The MetS criteria were in accordance with the NCEP:ATP III definition², which is having any 3 or more of the following 5 characteristics: BP \geq 130/85 mm Hg (either number, or use of antihypertensive medications); plasma glucose \geq 100 mg/dL; TG \geq 150 mg/dL, waist circumference (WC) \geq 102 cm for men or \geq 88 cm for women, and HDL-c $<$ 40 mg/dL for men or $<$ 50 mg/dL for women². Subjects had no current or history of heart disease, stroke, diabetes, liver disease, cancer, severe infections or autoimmune diseases. Subjects with

MetS were classified into two groups according to their HDL-c concentrations; Normal HDL-c group (n=23), (men=6; women=17), and Low HDL-c group (n=17), (men=5; women= 12).

*Normal HDL-c → men \geq 40 mg/dL and women \geq 50 mg/dL.

*Low HDL-c → men < 40 mg/dL and women < 50 mg/dL.

3.3 Anthropometrics

Weight in kilograms (kg) was measured to the closest 0.1kg on a portable scale and height in centimeters (cm) was measured to the closest 0.5 cm on a stadiometer. Both weight and height were used to calculate body mass index (BMI) in kg/m^2 as the following equation: $\text{BMI} = \text{weight (kg)} / \text{height (m)}^2$. WC was assessed with a flexible measuring tape that was placed at the top of the iliac crest directly on the skin, and subjects were standing forward, feet together, with both arms hanging freely. WC were averaged from 3 readings to the nearest 0.5 cm³. BP was measured on the right arm using an Omron automated BP cuff (Omron HEM 7320-Z/ HEM 7131-Z, Lake Forest, IL, USA), with subjects sitting quietly for 1-3 minutes rest. BP was measured from an average of 3 readings taken by the same individual to account for variability⁴.

3.4 Diet and Exercise Analysis

Diet Records Assessment was conducted through the analysis of 3-day diet records completed by participants at baseline. Participants were given instructions on how to fill out the diet records. Nutrition Data Systems for Research software (NDSR; 2013), developed by the Nutrition Coordinating Center, University of Minnesota, Minneapolis, MN, USA, was used to analyze the diet records⁸. Subjects completed a 3-day exercise records, and an average was calculated to use for analysis.

3.5 Blood Analysis

After a 12 hour overnight fast, blood samples were drawn from each subject. A total of 40 ml blood was collected from the antecubital vein into Ethylenediaminetetraacetic acid (EDTA)-containing tubes to prevent coagulation. A sample of whole blood (1ml) was collected and frozen at -80 degrees Celsius for further analysis. Plasma was isolated by centrifugation at 2000 x g for 20 minutes, and then 500 µl of plasma were used in Cobas c-111 analyzer (Roche Diagnostics, Indianapolis, IN)⁵ to determine plasma lipids, glucose, liver enzymes and C-reactive protein (CRP). The remaining plasma was aliquoted and frozen at -80 degrees C° for further analysis.

3.5.1 Lipid Analysis, Glucose, Glycosylated Hemoglobin (HbA1c), Liver Enzymes, C-Reactive Protein and Insulin

Plasma total cholesterol (TC), TG, glucose, HDL-c, CRP, liver enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST), and HbA1c were all determined using an automated clinical chemistry analyzer (Cobas c-111, Roche Diagnostics, Indianapolis, IN) via enzymatic reactions and photometric detection method⁵. Plasma LDL-c was calculated by the Friedewald equation: $LDL-c = TC - HDL-c - (TG/5)$. This equation was based on measurements of plasma TC, HDL-c and TG in mg/dL, and become the standard method for LDL-c assessment because it is simpler than direct assays⁶. Plasma insulin was measured by an ELISA kit (Crystal Chem, Elk Grove Village, IL), which utilizes a specific antibody immobilized onto the 96-microplate wells and an antibody labeled with HRP enzyme. After incubation and washout steps the substrate solution was added followed by incubation in the dark for 15 minutes. Then the stop solution was added and the insulin levels of samples were measured according color intensity by

using a BioTek Synergy 2 Multi-Mode Microplate Reader with Gen5 Software (BioTek Instruments, Inc.) at wavelength 450nm⁷.

3.5.2 Lipoproteins Size and Subfractions

Fasting plasma was used to determine total lipoprotein particle number, size, and concentration using proton nuclear magnetic resonance (NMR) spectroscopy. NMR analysis was performed on 400 MHz NMR analyzer (LipoScience, Inc., Raleigh, NC)⁸. This technique can quantify the size, number, and concentration of lipoprotein particles expressed each as an average particle size or as lipoprotein particle concentration by a specific methyl signal⁹. The NMR simultaneously quantifies >30 lipoprotein fractions that are grouped into subclasses based on size. The lipoprotein subfractions were: small VLDL (27–35 nm), medium VLDL (35–60 nm), large VLDL (>60 nm), IDL (23–27 nm), small LDL (18–19.8 nm), medium LDL (19.8–21.2 nm), large LDL (21.2–23 nm), small HDL (7.3–8.2 nm), medium HDL (8.2–8.8 nm), and large HDL (8.8–13 nm).

3.5.3 Apolipoprotein Analysis

Plasma apolipoproteins (apo A-I, apo A-II, apo B, apo C-II, apo C-III, and apo E) were quantified simultaneously by using a commercially human apolipoprotein multiplex assay kit (EMD Millipore, Billerica, MA) and analyzed by a Luminex MAGPIX analyzer (Luminex Corporation, Austin, Texas, United States)⁸. This procedure quantifies apolipoproteins in plasma using antibody-immobilized fluorescent dye labeled microspheres.

3.5.4 HDL Functionality: PON1 and SAA1

Plasma PON1 activity was measured by a commercial fluorometric assay kit (BioVision, Inc, Milpitas, CA)¹⁰. This assay kit enables rapid measurement of PON1 activity through utilizing

a fluorogenic substrate that is converted into a highly fluorescent product (Ex/Em = 368/460 nm). Plasma samples, standards, positive and negative controls were added to black 96- fluorescence microplate wells. The reactions were carried out according to the manufacturer's instructions and the fluorescence levels were measured in kinetic mode for 60 minutes at 37 degrees Celsius by BioTek Synergy H1 Microplate Fluorescence Reader with Gen5 Software (BioTek Instruments, Inc.; Winooski, VT, USA). Calculations were made by using the following formula:

$$\text{Sample PON1 Activity} = \frac{B}{\Delta T \times V} = \text{pmol/min/ml} = \text{U/ml}$$

Where: B = the amount of metabolite produced, calculated from the standard curve (in pmol)

ΔT = the linear phase reaction time $t_2 - t_1$ (in minutes)

V = the volume of sample added to the well (in ml)

Plasma SAA1 was measured by a ProcartaPlex Multiplex Immunoassay Human assay kit (Invitrogen, Vienna, Austria). This kit is used to perform quantitative multiplexed protein measurements in plasma samples using magnetic beads technology from Luminex to enable the simultaneous detection of SAA1¹¹. Samples and standards were added to the black 96- microplate. The protocol was followed according to the manufacturer's instructions and the samples were diluted 1:200. After the last incubation at room temperature on a plate shaker and the addition of reading buffer to all wells, the analysis was done by using a Luminex MAGPIX analyzer (Luminex Corporation, Austin, Texas, United States).

3.5.5 Plasma Inflammatory Biomarkers

Plasma tumor necrosis factor- α (TNF- α), monocyte chemoattractant protein-1 (MCP-1), Interleukin (IL)-6 and IL-8, were measured by use of the LINCOplex: Multiplex Biomarker Immunoassay for Luminex MAGPIX Technology (Luminex Corporation, Austin, Texas, United

States). This technique uses the fluorescently labeled microsphere beads with antibodies to each individual cytokine¹².

3.5.6 Plasma Antioxidant and Oxidative Stress Biomarkers and Oxidized LDL

Plasma glutathione peroxidase (GPx), superoxide dismutase (SOD), total antioxidant capacity (TAC), and catalase were measured using commercially available kits (Cayman Chemical Company, Ann Arbor, MI). The activity of GPx was measured indirectly by a coupled reaction with glutathione reductase¹³. SOD activity was determined by measuring the extent of superoxide radical generation by xanthine oxidase and hypoxanthine¹⁴. TAC was measured by assessing the oxidation extent of 2,2'-Azino-di-3-ethylbenzthiazoline sulfonate¹⁴. Catalase activity was assessed by measuring formaldehyde production in the presence of hydrogen peroxide. Thiobarbituric acid reactive substances (TBARS) were assessed as a marker of lipid peroxidation by using a commercially available kit (R&D Systems, Minneapolis, MN)¹⁵ and a BioTek Synergy 2 Multi-Mode Microplate Reader with Gen5 Software (BioTek Instruments, Inc.) for measuring the absorbance.

Ox-LDL was measured using a solid phase capture sandwich enzyme linked immunosorbent assay (ELISA) technology⁵. The 96-microwell plate to which the samples and standards were added was coated with antioxidantized LDL antibodies. After the washout step, HRP enzyme was added and then TMB substrates. TMB was catalyzed by HRP to produce a blue color that changed into yellow after adding the stop solution. The intensity of the yellow color is directly proportional to the concentration of Ox-LDL on the plate. The Ox-LDL concentrations were measured by using a BioTek Synergy 2 Multi-Mode Microplate Reader with Gen5 Software (BioTek Instruments, Inc.) at wavelength: 450nm.

3.6 Statistical Analysis

Statistical analysis was performed using SPSS version 25 statistical software for Windows (SPSS, Inc., Chicago, IL). Significance was defined as $p < 0.05$. Data values were represented as mean \pm standard deviation (SD). Un-paired t tests were used to determine significant differences between the groups in plasma lipids, apoproteins, lipoprotein subfractions, plasma inflammatory biomarkers, plasma antioxidant biomarkers and anthropometric measurements. Pearson correlations were used to evaluate positive and/or negative relationships between HDL level and lipoprotein size and number.

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Chapter 4

Results

4.1 Baseline Characteristics

To assess whether low HDL-c concentrations are associated with the biomarkers of cardiovascular disease, 40 subjects who classified with MetS according to the NCEP:ATP III criterion were divided into two groups based on plasma HDL-c concentrations into Normal HDL-c [men ≥ 40 mg/dL and women ≥ 50 mg/dL] and Low HDL-c [men < 40 mg/dL and women < 50 mg/dL]. Subject's diet intake and exercise records are shown in **Table 1**. There were no significant differences between the two groups in any of the analyzed nutrients including macronutrients, types of fat, glycemic index, fiber or carotenoids. Similarly, there were not significant differences in terms of minutes exercise per day in both groups. Subject's age ranged from 32 -70 years old and their anthropometric measurements are displayed in **Table 2**. Based on BMI values, the majority of subjects were in the obese category (n=27). WC on average was 106.9 cm for the Normal HDL-c group and 110 cm for the Low HDL-c group. Plasma HDL-c on average was 61.4 mg/dL in subjects with Normal HDL-c and 38.5 mg/dL in subjects with Low HDL-c. Systolic blood pressure (BP) as well as TG were different between groups. The Low HDL-c group had significantly higher systolic BP and TG than Normal HDL-c group ($p < 0.01$). Other MetS parameters (WC, fasting glucose and diastolic BP) were not different between groups. A negative correlation was found between HDL-c and waist circumference (WC) ($r = -.0418$, $p < 0.01$) and between HDL-c and insulin ($r = -0.413$, $p < 0.025$) as presented in **Figure 1** and **Figure 2**, respectively. In addition, a positive correlation was found between TG and insulin values as presented in **Figure 3**.

Table 1. Diet and exercise records in Normal HDL-c and Low HDL-c groups

| Parameter | Normal HDL-c | Low HDL-c | P value |
|------------------------------|-----------------|-----------------|---------|
| Total Energy (kcal) | 2051.4 ± 891.6 | 2025.1 ± 472.3 | 0.913 |
| Carbohydrate (%) | 38.3 ± 9.0 | 40.4 ± 6.7 | 0.408 |
| Protein (%) | 17.2 ± 2.9 | 18.3 ± 3.8 | 0.315 |
| Fat (%) | 41.2 ± 8.6 | 39.1 ± 7.4 | 0.428 |
| SFA (g/day) | 30.3 ± 12.2 | 31.4 ± 15.1 | 0.797 |
| MUFA (g/day) | 39.7 ± 41.8 | 32.9 ± 11.9 | 0.523 |
| PUFA (g/day) | 21.3 ± 17.5 | 19.5 ± 6.4 | 0.676 |
| Trans Fatty Acids (g/day) | 2.5 ± 2.3 | 2.1 ± 0.94 | 0.250 |
| Cholesterol (mg/day) | 311.0 ± 139.7 | 311.7 ± 193.2 | 0.990 |
| Total Fiber (g/day) | 21.8 ± 14.8 | 24.1 ± 10.3 | 0.590 |
| Soluble Fiber (g/day) | 6.7 ± 2.2 | 7.7 ± 2.4 | 0.224 |
| Insoluble Fiber (g/day) | 14.9 ± 13.5 | 16.4 ± 8.5 | 0.700 |
| Glycemic Index | 57.7 ± 6.1 | 56.6 ± 5.6 | 0.577 |
| Glycemic Load | 101.7 ± 44.2 | 106.4 ± 34.5 | 0.720 |
| β-Carotene (μg) | 3423.4 ± 3255.9 | 3886.2 ± 3430.1 | 0.666 |
| α-Carotene (μg) | 577.7 ± 1045.2 | 645.8 ± 924.4 | 0.832 |
| Lycopene (μg) | 6522.7 ± 7733.0 | 3959.8 ± 4016.2 | 0.221 |
| Lutein + Zeaxanthin (μg/day) | 2390.8 ± 2108.9 | 3830.7 ± 4787.4 | 0.206 |
| Exercise (minutes/week) | 36.7 ± 29.8 | 53.8 ± 50 | 0.185 |

- Data presented as Mean ± SD

Table 2. Anthropometrics, blood pressure (BP), plasma total cholesterol (TC), triglycerides (TG), HDL-c, LDL-c, plasma glucose, plasma insulin and glycosylated hemoglobin (HbA1c) in Normal HDL-c and Low HDL-c groups

| Parameter | Normal HDL-c | Low HDL-c | P value |
|-------------------------------|---------------------|---------------------|---------|
| Age (years) | 53.3 ± 9.4 | 51.2 ± 9.8 | 0.492 |
| Weight (kg) | 88.6 ± 17.3 | 93.6 ± 14.6 | 0.341 |
| BMI (kg/m ²) | 32 ± 4.1 | 32.7 ± 3.3 | 0.563 |
| WC (cm) | 114.2 ± 15.5 (men) | 113.6 ± 11.5 (men) | 0.948 |
| | 104.4 ± 8.3 (women) | 108.5 ± 9.5 (women) | 0.231 |
| WC (cm) Both genders | 106.9 ± 11.2 | 109 ± 10.1 | 0.381 |
| Systolic BP (mm Hg) | 121.3 ± 14.2 | 129.9 ± 9.8 | 0.028 |
| Diastolic BP (mm Hg) | 84.8 ± 7.1 | 83.1 ± 9.1 | 0.507 |
| TC (mg/dL) | 187.4 ± 36.3 | 178.6 ± 30.8 | 0.426 |
| TG (mg/dL) | 115.5 ± 56.8 | 165.7 ± 80.7 | 0.026 |
| HDL-c (mg/dL) | 47.2 ± 5.1 (men) | 31.6 ± 3.8 (men) | 0.000 |
| | 66.5 ± 18.6 (women) | 41.4 ± 3.8 (women) | 0.000 |
| HDL-c (mg/dL) Both genders | 61.4 ± 18.2 | 38.5 ± 5.9 | 0.000 |
| LDL-c (mg/dL) | 102.7 ± 31.7 | 107 ± 29.6 | 0.662 |
| Glucose (mg/dL) | 106.2 ± 10.1 | 105.1 ± 9.9 | 0.733 |
| Insulin (pmol/L) | 45.6 ± 29.9 | 60.3 ± 29.1 | 0.141 |
| HbA1c (%) | 5.6 ± 0.45 | 5.6 ± 0.58 | 0.815 |

- Data presented as Mean ± SD

Figure 1. Negative correlation between HDL-c concentrations and waist circumference (WC)

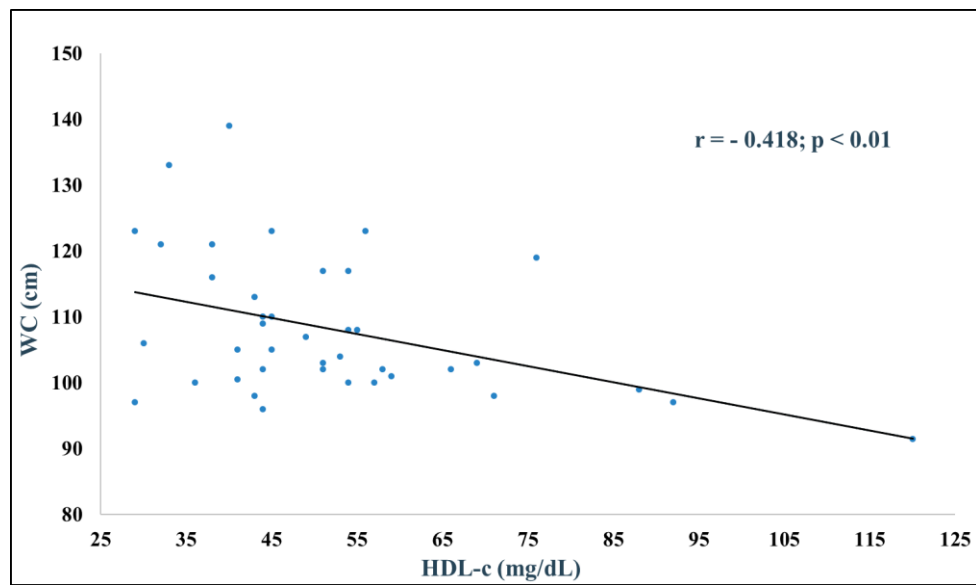


Figure 2. Negative correlation between HDL-c concentrations and insulin

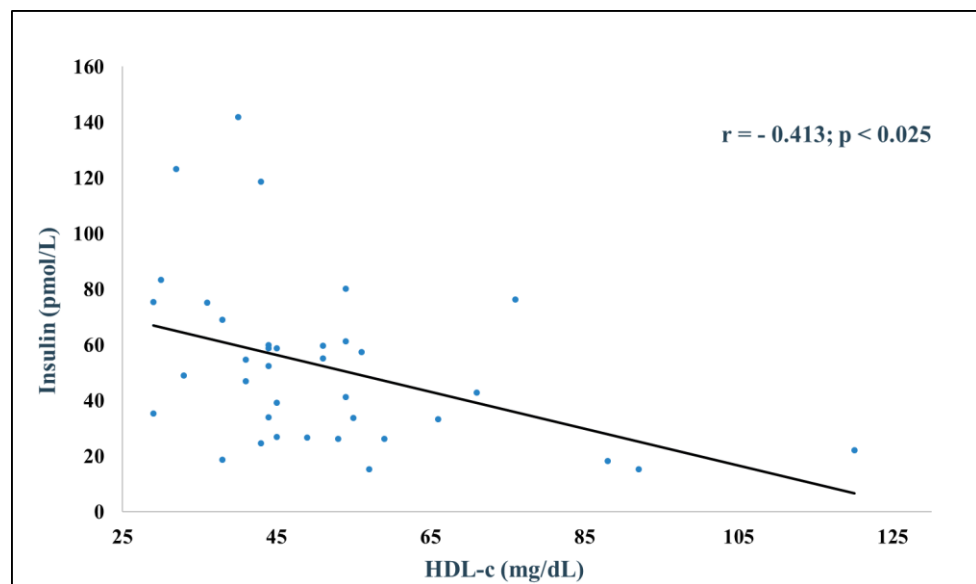
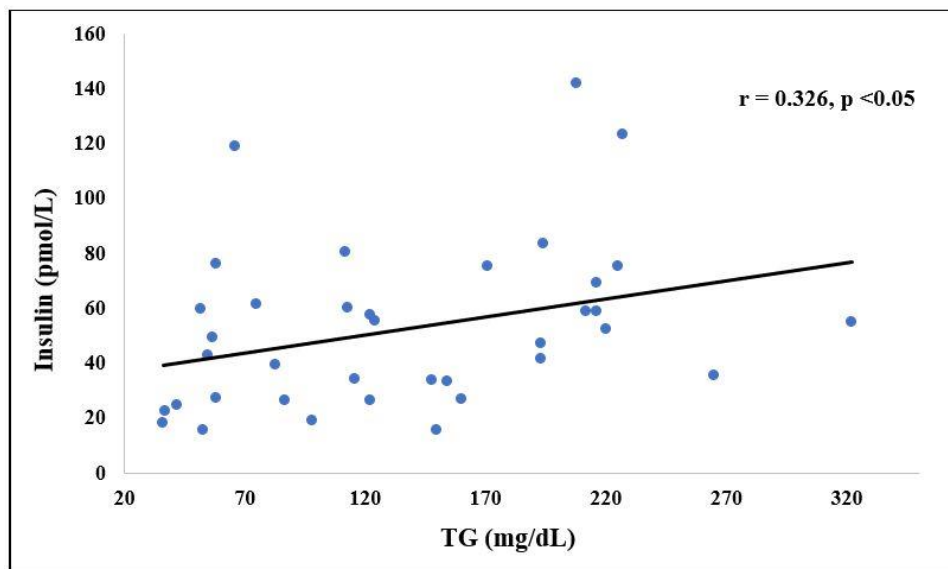


Figure 3. Positive correlation between TG levels and insulin



4.2 Lipoprotein Size and Subfractions

VLDL, LDL and HDL size as well as VLDL, LDL and HDL subfractions were assessed using NMR spectroscopy. Numbers, size, and total of each lipoprotein. Total VLDL particles in Normal HDL-c group and Low HDL-c group are presented in **Figure 4**. Large and medium sizes of VLDL particles were significantly higher in the Low HDL-c group [large: $p=0.028$; medium: $p=0.005$] as presented in **Figure 5**. In addition, total LDL particles in Normal HDL-c group and Low HDL-c group are presented in **Figure 6**. As shown in **Figure 7**, large LDL particles, which are the less atherogenic lipoprotein were lower in the Low HDL-c group while small LDL particles, which are known to be the most atherogenic lipoprotein were higher when compared with Normal HDL-c [large LDL: $p=0.012$, and small LDL: $p=0.002$]. Total HDL particles were lower significantly in the Low HDL-c group compared to the Normal HDL-c group ($p=0.000$), as shown in **Figure 8**. In addition, HDL particles distributions are presented in **Figure 9**. Large HDL particles were lower significantly in the Low HDL-c group compared to the Normal HDL-c group ($p<0.05$). LDL and HDL particles as measured in nanometers were lower in the Normal HDL-c group compared to the Low HDL-c group as presented in **Figure 10**.

Figure 4. Total VLDL particles (nmol/L) in Normal HDL-c and Low HDL-c groups

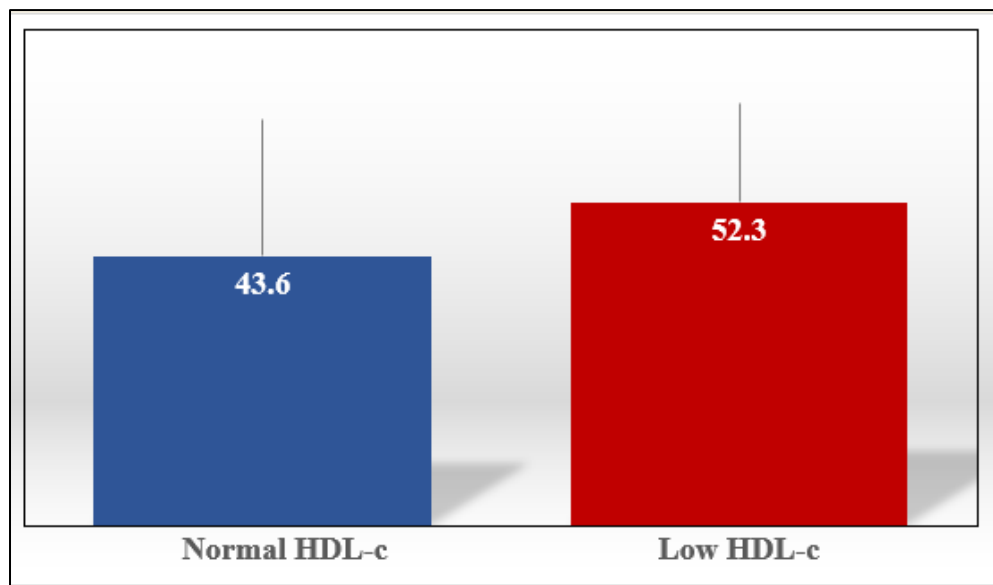


Figure 5. Concentrations of large, medium and small VLDL (nmol/L) in Normal HDL-c and Low HDL-c groups

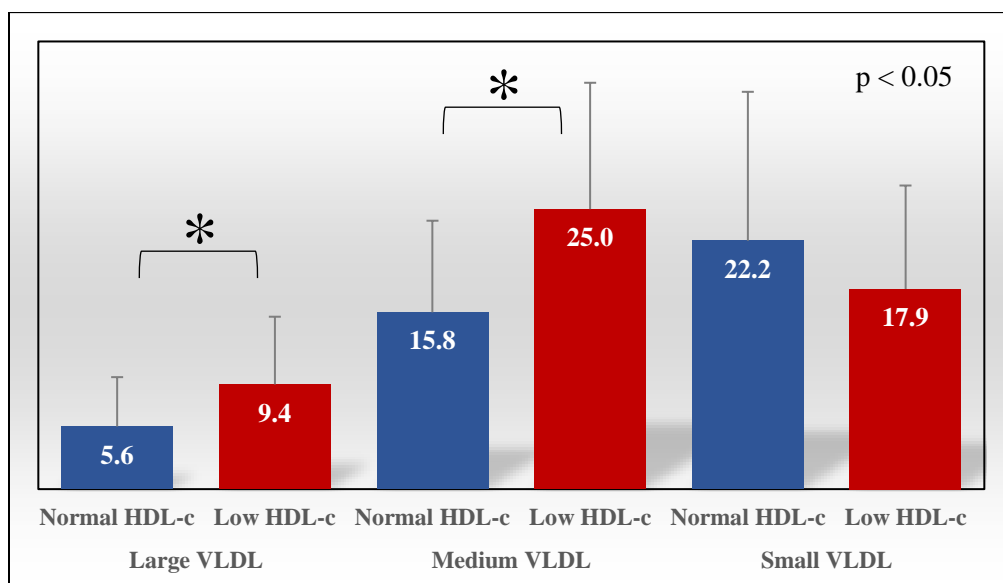


Figure 6. Total LDL particles (nmol/L) in Normal HDL-c and Low HDL-c groups

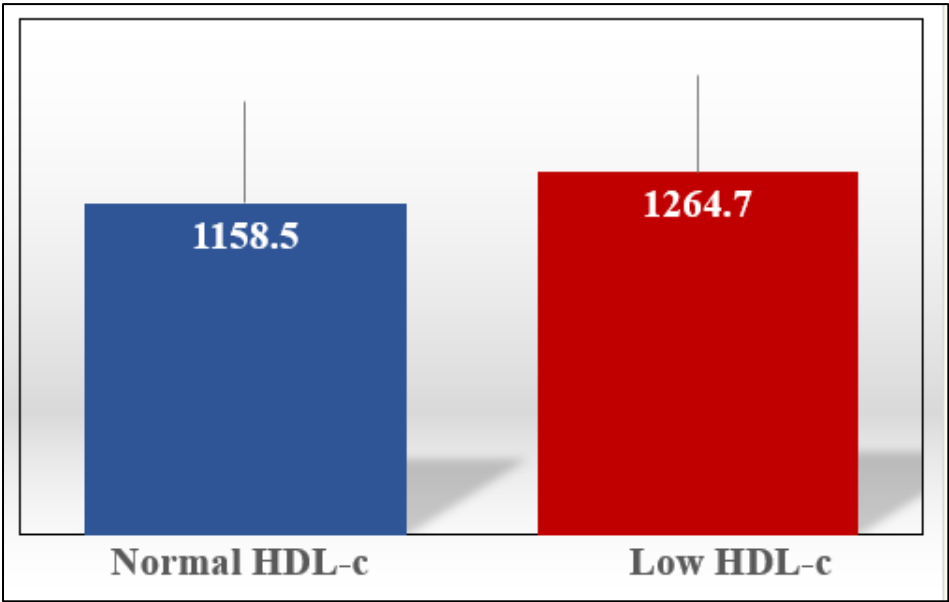


Figure 7. Concentrations of large and small LDL (nmol/L) in Normal HDL-c and Low HDL-c groups

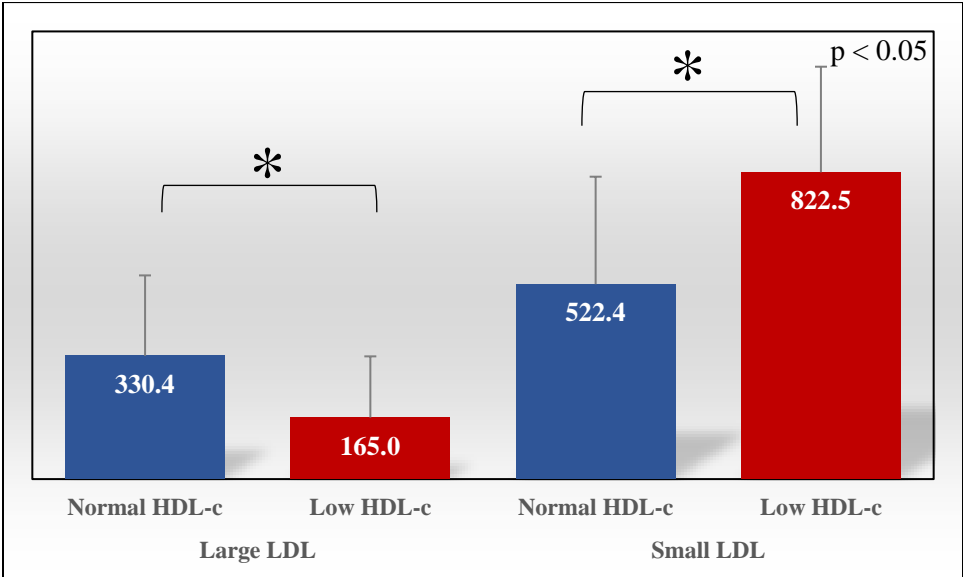


Figure 8. Total HDL particles ($\mu\text{mol/L}$) in Normal HDL-c and Low HDL-c groups

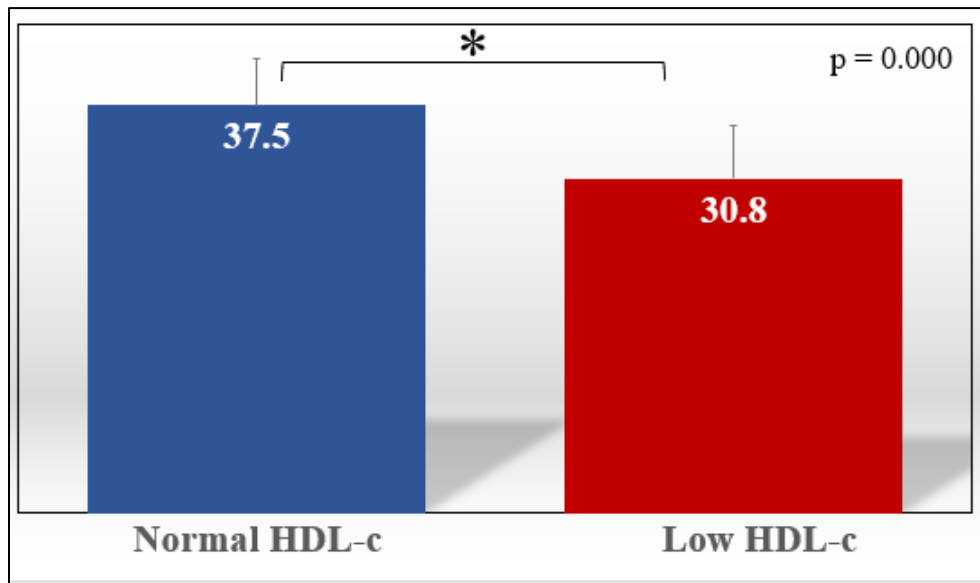


Figure 9. Concentrations of large, medium and small HDL ($\mu\text{mol/L}$) in Normal HDL-c and Low HDL-c groups

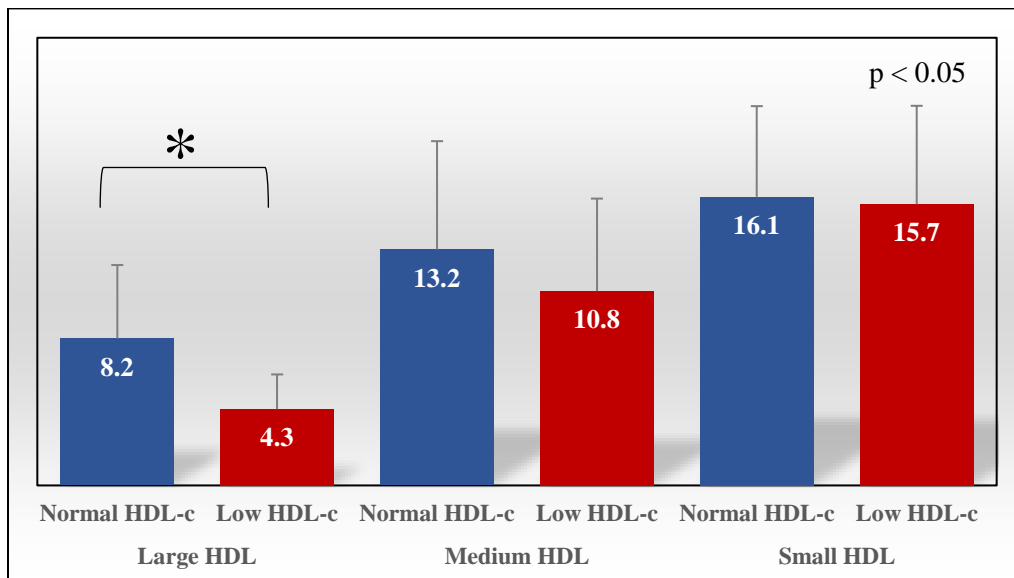
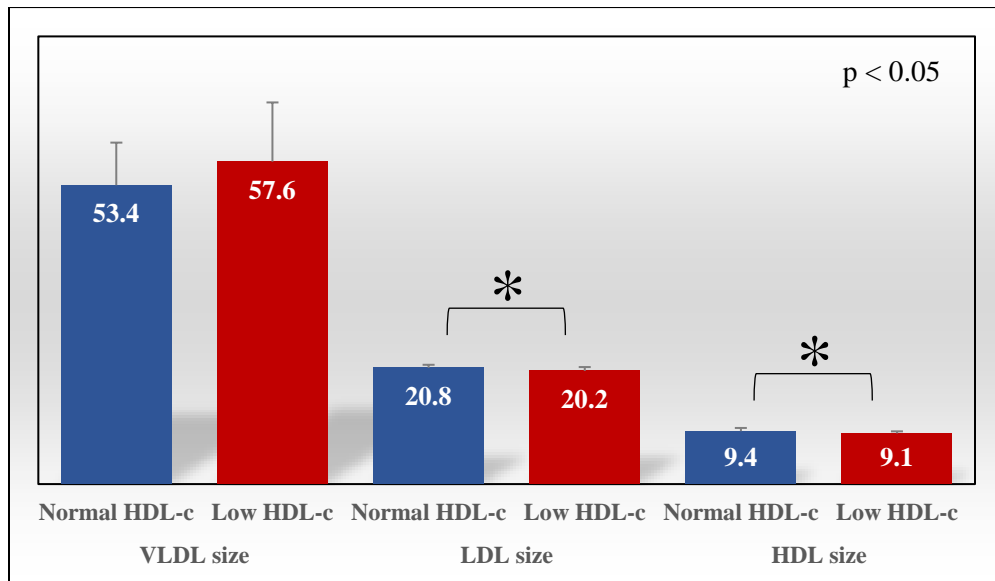


Figure 10. Lipoprotein size (nm) in Normal HDL-c and Low HDL-c groups



4.3 Apolipoproteins (apo) Distribution based on Plasma HDL-c

The distribution of apo A-I, apo A-II, apo C-II, apo C-III, apo E, and apo B in the plasma was assessed using a human apolipoprotein multiplex assay kit to compare apolipoproteins distribution between the two groups as presented in **Table 3**. Apo A-I was significantly lower in Low HDL-c group compared to the Normal HDL-c group. None of the other apolipoproteins were statistically significant between groups. There was positively correlation between Apo A-I and large HDL particles that presented in **Figure 11**. Also, there was a positive correlation between Apo A-I and total HDL in **Figure 12**. When waist measurement increased, apo A-I is that mainly present in HDL particles was decreased that was presented as a negative correlation between WC and apo A-I in **Figure 13**. There was a positive correlation between TG and apo C-III that presented in **Figure 14**.

Table 3: Apolipoproteins distribution based on plasma HDL-c

| Parameter | Normal HDL-c | Low HDL-c | P value |
|------------------|----------------|----------------|---------|
| Apo A-I (mg/L) | 985.4 ± 274.2 | 790.7 ± 188.1 | 0.019 |
| Apo A-II (mg/L) | 599.0 ± 159.4 | 586.4 ± 173.9 | 0.813 |
| Apo C-II (mg/L) | 243.4 ± 161.1 | 283.8 ± 212.5 | 0.497 |
| Apo C-III (mg/L) | 474.7 ± 274.1 | 520.1 ± 330.8 | 0.639 |
| Apo E (mg/L) | 106.2 ± 60.4 | 125.3 ± 62.9 | 0.339 |
| Apo B (mg/L) | 1707.1 ± 704.4 | 1908.8 ± 598.0 | 0.346 |

- Data presented as Mean ± SD

Figure 11. Positive correlation between large HDL and Apo A-I

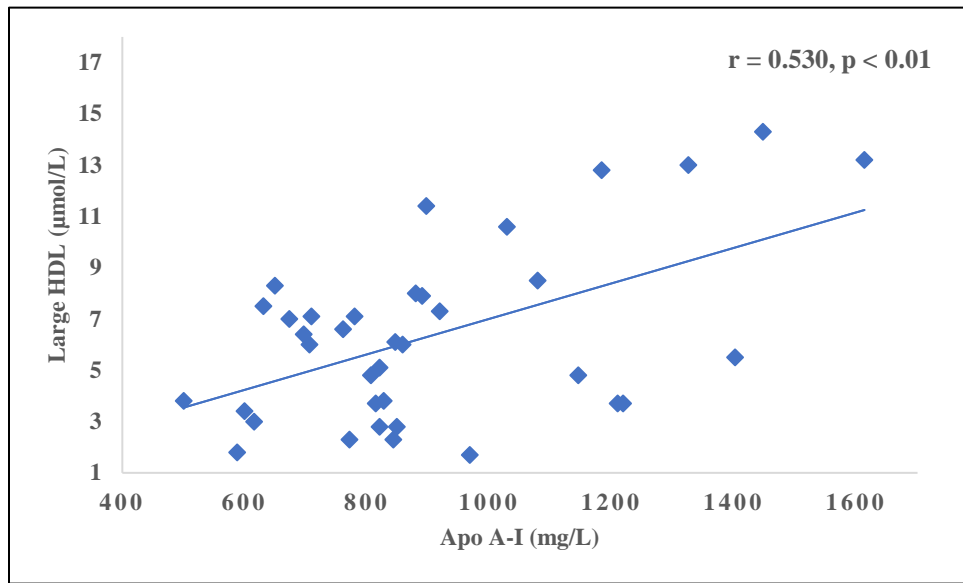


Figure 12. Positive correlation between total HDL and Apo A-I

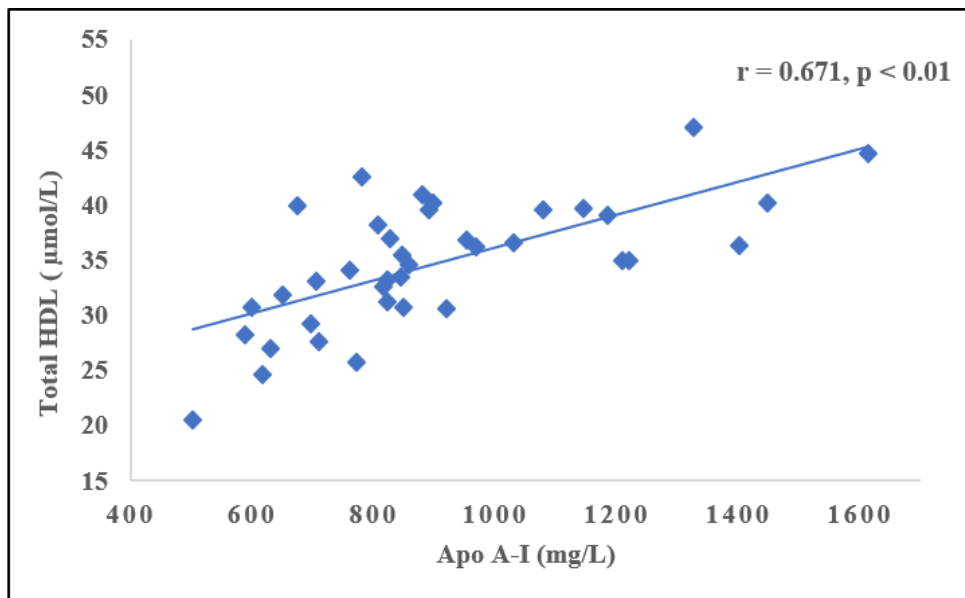


Figure 13. Negative correlation between WC and Apo A-I

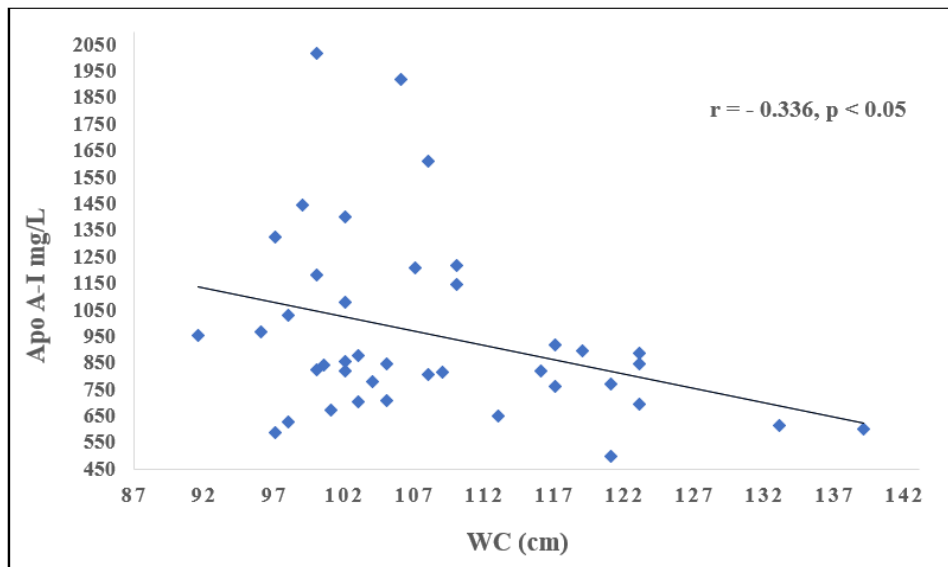
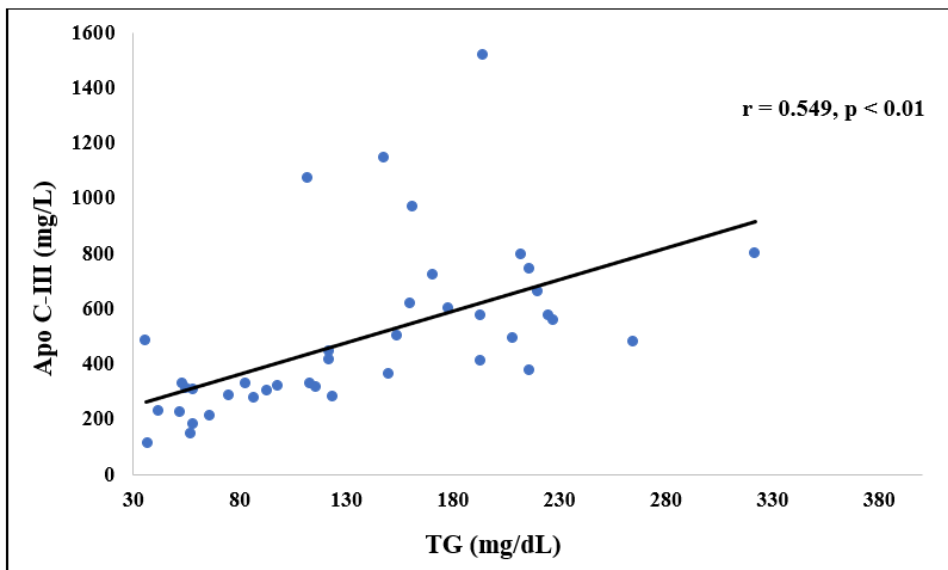


Figure 14. Positive correlation between TG and Apo C-III



4.4 HDL Functionality [PON-1 and SAA1]

PON-1 was assessed as a functional marker for HDL particles, its activity was measured by using a fluorometric assay kit. PON-1 activity values for both groups are presented in **Figure 15**. PON-1 values were significantly higher in the Normal HDL-c compared to the Low HDL-c group ($p = 0.022$). In **Figure 16**, a positive correlation was found between PON-1 and total HDL. SAA1 was measured as a functional marker for HDL. In **Figure 17**, SAA1 values are presented for both Normal HDL-c group and Low HDL-c group. There were no significant differences between groups. In addition, there was a positive correlation between SAA1 and the inflammatory marker CRP that is presented in **Figure 18**.

Figure 15. Paraoxonase-1 (PON-1) activity in Normal HDL-c and Low HDL-c groups

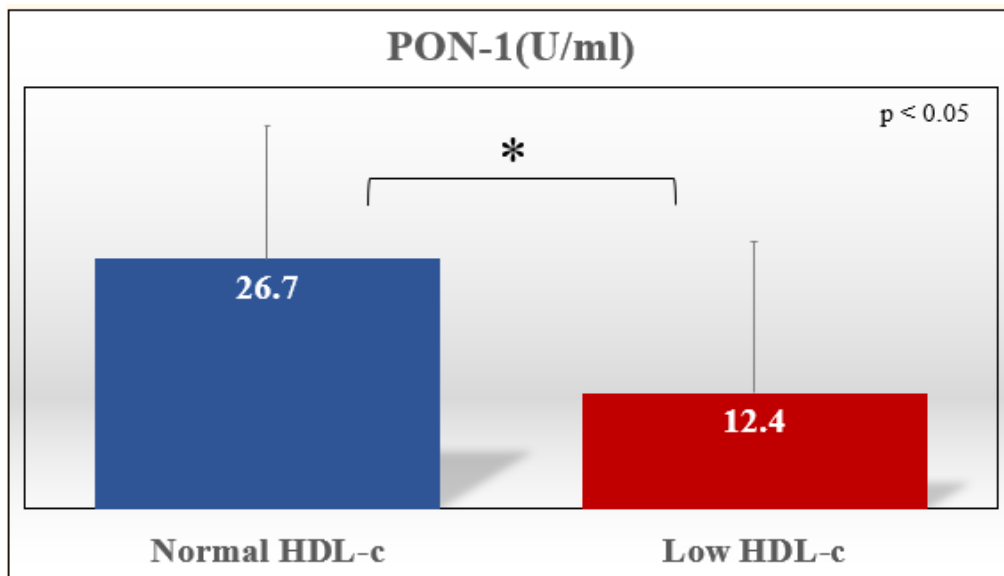


Figure 16. Positive correlation between PON-1 and total HDL

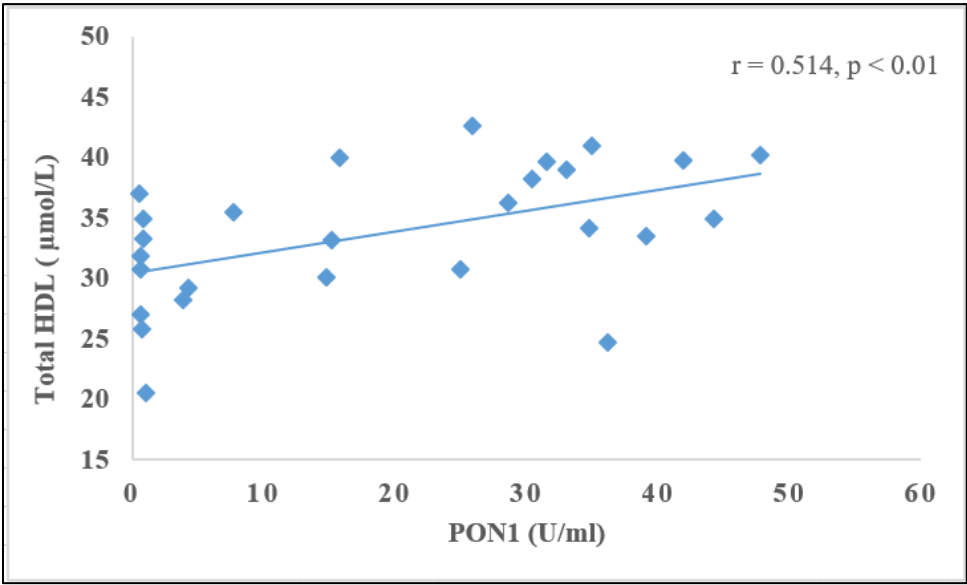


Figure 17. Serum Amyloid A1 (SAA1) in Normal HDL-c and Low HDL-c groups

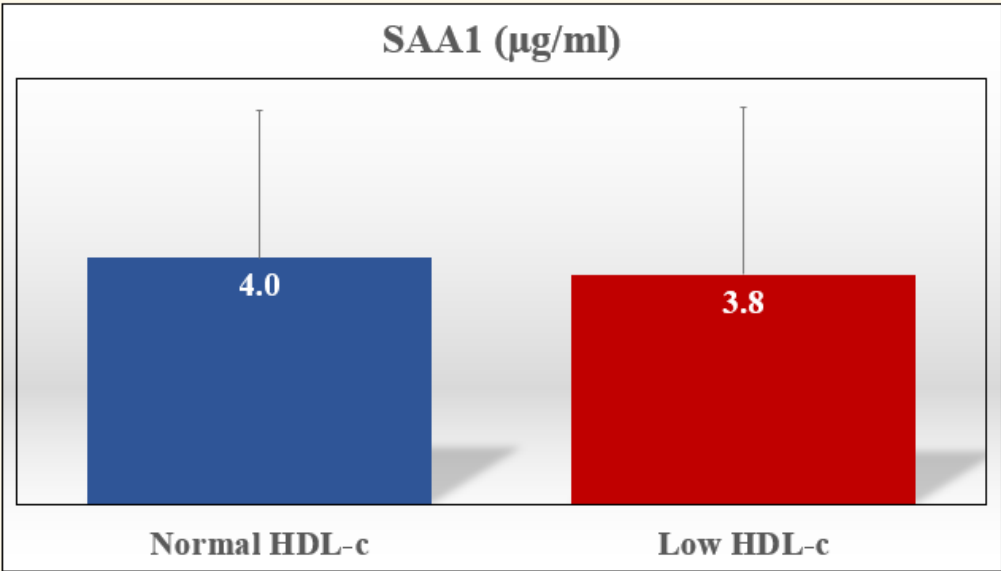
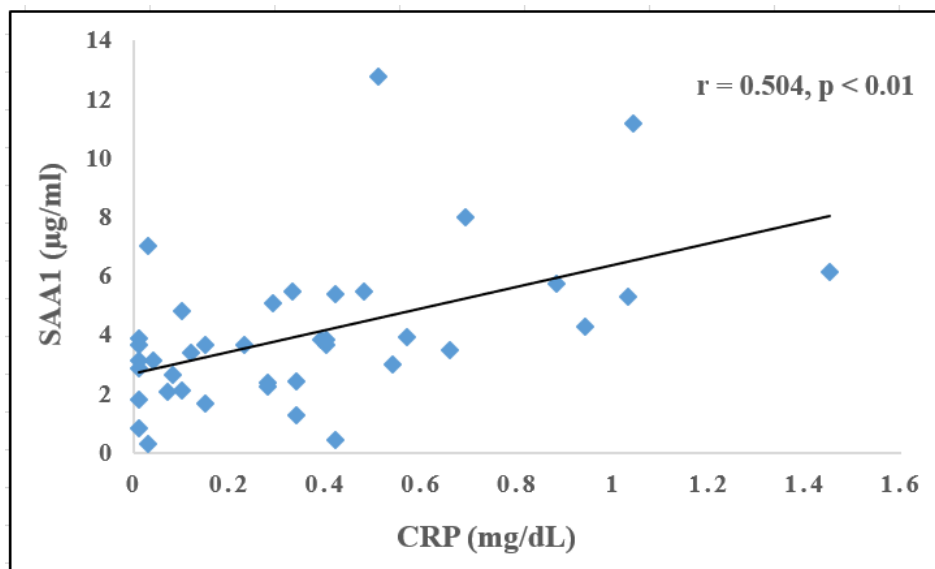


Figure 18. Positive correlation between CRP and SAA1



4.5 Inflammatory Markers

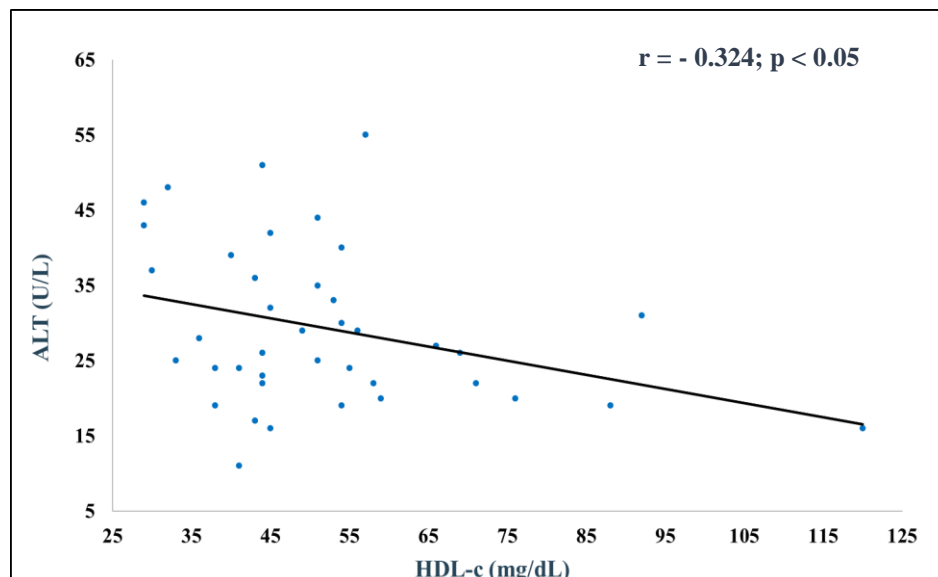
Mean values for inflammatory markers [liver enzymes [alanine aminotransferase (ALT) and aspartate aminotransferase (AST)], C-reactive protein (CRP), tumor necrosis factor- α (TNF- α), monocyte chemoattractant protein-1 (MCP-1), and Interleukin (IL)-6 and IL-8] categorized for Normal HDL-c and Low HDL-c groups are presented in **Table 4**. None of the inflammatory markers were significantly different between groups. There was a negative correlation between HDL-c and ALT, which is one of the liver enzymes as shown in **Figure 19**.

Table 4. Inflammatory markers and liver enzymes in Normal HDL-c and Low HDL-c groups

| Parameter | Normal HDL-c | Low HDL-c | P value |
|-----------------------|------------------|------------------|---------|
| AST (U/L) | 25.0 \pm 6.1 | 26.2 \pm 7.4 | 0.598 |
| ALT (U/L) | 29.2 \pm 10.7 | 29.6 \pm 10.8 | 0.915 |
| CRP (mg/dL) | 0.39 \pm 0.39 | 0.31 \pm 0.29 | 0.456 |
| TNF- α (pg/mL) | 6.2 \pm 2.4 | 6.9 \pm 3.9 | 0.508 |
| MCP-1 (pg/mL) | 129.6 \pm 66.6 | 122.2 \pm 29.0 | 0.676 |
| IL-6 (pg/mL) | 5.5 \pm 0.78 | 5.99 \pm 1.3 | 0.185 |
| IL-8 (pg/mL) | 9.7 \pm 10.3 | 7.9 \pm 1.2 | 0.479 |

- Data presented as Mean \pm SD

Figure 19. Negative correlation between HDL-c and ALT



4.6 Antioxidants and Oxidative Stress Biomarkers and Oxidized LDL

Plasma antioxidant and oxidative stress biomarkers [thiobarbituric acid reactive substances (TBARS), total antioxidant capacity (TAC), superoxide dismutase (SOD), plasma glutathione peroxidase (GPx), catalase (CAT)] as well as Ox-LDL are presented in **Table 5**. Total antioxidant capacity (TAC) was significantly higher in subjects with Normal HDL-c ($p = 0.044$) when compared to subjects with Low HDL-c. Other markers were not different between groups.

Table 5. Antioxidants and oxidative stress biomarkers and oxidized LDL (Ox-LDL) in Normal HDL-c and Low HDL-c groups

| Parameter | Normal HDL-c | Low HDL-c | P value |
|-----------------------------|------------------|------------------|---------|
| TBARS (μM) | 0.16 ± 0.03 | 0.14 ± 0.03 | 0.087 |
| TAC (mM Trolox equivalents) | 2.1 ± 1.5 | 1.2 ± 0.9 | 0.044 |
| SOD (U/mL) | 2.3 ± 1.2 | 1.9 ± 1.2 | 0.361 |
| GPx (nmol/min/mL) | 150.6 ± 23.9 | 141.5 ± 20.2 | 0.220 |
| CAT (nmol/min/mL) | 19.4 ± 11.7 | 17.2 ± 7.0 | 0.507 |
| Ox-LDL (ng/ml) | 201.2 ± 14.5 | 212.6 ± 62.9 | 0.405 |

- Data presented as Mean \pm SD

Chapter 5

Discussion and Conclusion

Metabolic Syndrome is a well-defined condition that increases the risk of CVD and T2D¹. We assessed data collected from 40 individuals, 11 men and 29 women classified with MetS, by separating subjects into two categories based on HDL-c concentrations. This classification allowed us to determine whether low HDL-c is associated with additional biomarkers of CVD such as a high atherogenic dyslipidemia, a low grade of inflammation, and high oxidative stress biomarkers. In addition, this classification allowed us to understand the importance of measuring HDL functionality along with measuring plasma HDL-c to identify risk factors of CVD among participants with MetS.

In this study we found that individuals with MetS and low HDL-c had higher TG, systolic BP, WC, and lower total antioxidant capacity than those with normal HDL-c. We also found that subjects with low HDL-c had a more atherogenic lipoprotein profile as assessed by higher concentrations of large VLDL, medium VLDL and small LDL. In terms of HDL functionality, we found that Apo A-I was positively correlated with both large HDL and total HDL particles. Also, Apo A-I was significantly lower in Low HDL-c group. Further, we found a diminished HDL functionality as determined by PON-1 activity.

5.1 Metabolic Syndrome Parameters

Obesity is a crucial characteristic that increases the metabolic complications among individuals with MetS. Increases in weight and BMI are strongly associated with health issues for example: CVD, insulin resistance, T2D, hyperlipidemia, and hypertension². Unhealthy diets and

physical inactivity are the common habits that accelerate obesity rate³. These two factors were shown in the subject's diet and exercise records as high caloric intake, high saturated fatty acids intake, and low physical activity level. As demonstrated in this study and based on BMI categories, all subjects in this study were either overweight or obese, and more than half of them fit the obese categories including obese I [BMI= 30.0 – 34.9 kg/m²], obese II [BMI= 35.0 – 39.9 kg/m²]. The main problem with obesity is fat distribution and fat deposition⁴. Excess adipose tissues are associated with altering adipokines production, and that might increase the risk of insulin resistance as well as CVD⁴. Particularly, visceral adiposity is strongly correlated with insulin resistance⁴. Visceral adiposity can be measured through measuring the waist circumference area. WC was the most common MetS parameter determined in this population. Palaniappan et al. have shown that each 11 cm increase in WC is associated with an adjusted 80% increased risk of developing MetS condition⁵. In addition, according to the NCEP:ATP III, abdominal obesity is known to be an essential characteristic of MetS⁶. All participants in the current study met the WC requirement, in addition to two other markers of MetS diagnosis criterion. Another marker of MetS is high BP. The mean of systolic BP was significantly increased among Low HDL-c group. Elevated BP is known to be one of the CVD risk factors, and it was estimated that 5 mm Hg reduction in systolic BP would result in overall reduction of 14% in stroke mortality and 9 % in CHD mortality⁴. In a study done on subjects with MetS, approximately 25.1% had high TG levels (TG > 150 mg/dL)². In the present study, 48% of subjects had high TG levels. Plasma TG concentrations were significantly different between the two groups. The Low HDL-c group had higher TG values than subjects in the Normal HDL-c group. Elevated TG values are strongly correlated with increased risk of CVD. Insulin resistance results either from a genetic defect or obesity, and it may lead to elevation in TG and fasting glucose levels as well as BP and reduction

of HDL-c⁵, as demonstrated in this study. Elevated TG was positively correlated with insulin levels. In addition, measuring fasting glucose in obese subjects is an important predictor for other CVD risk factors, and it is one of the MetS parameters. Since the majority of the current subjects fit the obese category, 48% of them had hyperglycemia that placed them in MetS condition. Since subjects were divided into two groups based on their HDL-c values, HDL-c concentrations were significantly different between the two groups and between genders in each group. Low HDL-c values were always linked to be a risk factor of CVD and T2D⁷. Observational studies have shown that each decrease of 1-mg/dL in HDL-c concentration is associated with a 2% - 3% increased risk of CVD^{8,9}. In contrast, normal HDL-c concentrations are recommended and linked to be a protective parameter against diseases.

5.2 Lipoprotein Particles

Recently, interest has shifted from not only focusing on the quantity of HDL particles but also focusing on HDL quality⁹. HDL exhibits antiatherogenic mechanisms including its role in the RCT process, anti-inflammatory, antioxidant and antithrombotic functions, all of which could contribute to the protection against atherosclerosis¹⁰. These functions may be disrupted when HDL particles become dysfunctional. Therefore, measuring lipoprotein particle size and number is an important aspect to better understand lipoproteins metabolism¹¹. Lipoproteins particles that are considered atherogenic for example: large VLDL, medium VLDL, and small LDL particles are present in increased numbers in the MetS populations¹². The number of these particles has been shown to be positively associated with CVD risk. In the current study, NMR method was used to measure number and size of different lipoprotein particles. This method not only quantifies the overall lipoprotein particles, but also measures different sizes for each lipoprotein. The NMR method is well known as a useful and accurate method that helps to link specific lipoproteins to

CVD¹¹. Our findings indicate that MetS subjects with Low HDL-c had significantly increased numbers of large and medium VLDL as well as of small LDL compared to subjects with Normal HDL-c while large LDL particles were lower in numbers compared with the Normal HDL-c group. Large LDL particles are known to be less susceptible to oxidation and subendothelial retention than small LDL particles¹³. Calculating LDL-c did not give us enough information about the whole picture of the LDL particles. Thus, measuring LDL particles subfractions provided important information in regards to which particles were dominant as well as it allowed us to compare these particles across subjects with MetS based on the HDL-c values. That also allowed us to draw a conclusion that low HDL-c is associated with more atherogenic lipoprotein profiles. In contrast, large HDL particles were significantly higher among subjects in the Normal HDL-c group also indicating a more efficient RCT in this group and in addition negative correlation have been found between HDL particles numbers and CVD¹⁴.

5.3 Apolipoproteins

In regards to apolipoproteins, Apo A-I was significantly higher in the Normal HDL-c group compared to the Low HDL-c group. Apo A-I is mainly present in HDL particles and higher levels of this apolipoprotein were inversely associated with CVD risk. According to the current data, there was a positive correlation between Apo A-I and both large HDL and total HDL. Also, an inverse correlation was found between WC and apo A-I. As mentioned previously, obesity particularly the increase in WC, has effects on well-being, and is considered an independent risk factor for CVD. HDL-c has been shown to improve through weight loss including decreases in WC¹⁵. Our findings indicate that when WC increased apo A-I was decreased suggesting that HDL might improve with decreases in weight and WC. Even though subjects in the Normal HDL-c group has higher values of HDL-c, that does not necessary means the presence of functional HDL

particles. Thus, measuring HDL functionality markers are important to provide a better view of the atheroprotective lipoprotein HDL. HDL particles may have proatherogenic modifications that lead to increase the risk of chronic diseases instead of being protective. Dysfunctional HDL result in decreases in cholesterol efflux and increases in inflammation and thrombosis. There are some markers indicative of HDL dysfunctionality, such as, apo C-III and SAA1. In the current study, apo C-III was positively correlated with TG levels. It is known that apo C-III has proinflammatory and prothrombotic effects⁹. HDL functionality can be evaluated through measuring apo A-I, and PON-1. In the current study, Apo A-I was significantly lower in the Low HDL-c group in compared to the Normal HDL-c group.

5.4 HDL Functionality [PON-1 and SAA1]

The enzyme that associates with HDL particles and has atheroprotective functions is PON-1. Lower PON-1 activity is related to CHD, and the MetS condition has been shown to reduce the enzymatic capacity of PON-1¹⁶. In the current study, plasma PON-1 was measured in kinetic mode by fluorometric assay kit. Subjects with Normal HDL-c had significantly higher PON-1 activity compared to subjects with Low HDL-c. Increased PON-1 activity is a marker for HDL functionality, and Normal HDL-c group had higher numbers of large HDL particles and HDL-c concentrations. In addition, PON-1 activity levels were positively correlated with total HDL, and with large HDL particles. Thus, large HDL particles seem to be functional in Normal HDL-c group. One study has shown that PON-1 activity levels are significantly lower in MetS subjects¹⁶. That finding was also demonstrated in the present study.

Another marker for HDL functionality is SAA1. SAA1 is an apolipoprotein of HDL in the acute-phase, which causes HDL remodeling and dysfunctionality¹⁷. It was documented that SAA1 plays a major role in lipid metabolism because it can alter the distribution of cholesterol in the

lipoproteins, and can be act as a lipid acceptor for ABCA1 but its impact on lipid metabolism remains incompletely understood¹⁷. SAA1 for this study was measured in plasma samples, and there was no statically difference for SAA1 between the two groups. SAA1 increases in acute phases, and we found that SAA1 values were positively correlated with the inflammatory marker CRP. It is well established that elevated CRP levels are associated with the increase of WC, BMI and hyperlipidemia along with MetS condition⁴. That was also documented in the current study.

5.5 Inflammatory and Antioxidants Biomarkers

Individuals with MetS are often obese, and obesity is known to be associated with low-grade inflammation and increased oxidative stress ¹⁸. Studies have shown that MetS has independently linked with low-grads inflammation and increased oxidative stress biomarkers. Also, plasma TNF- α is positively associated with body weight, WC, and TG, while it is negatively associated with HDL-c⁴. Liver enzymes are commonly used as indicators for liver functions. Studies have shown that individuals with MetS have higher values of liver enzymes [ALT and AST] compared with healthy populations¹⁹. Our findings indicate that HDL-c values in this population were negatively associated with the liver enzyme ALT. While there was no significant difference in inflammatory markers between groups, total antioxidant capacity was significantly different. Because antioxidants are mostly carried in the body through HDL, the increase of HDL will help in the transportation of these antioxidants throughout the body as, was demonstrated in this study. TAC values for our subjects were significantly higher among subjects in the Normal HDL-c group. On the other hand, lipid peroxidation markers such as Ox-LDL are increased in MetS²⁰. Both lipid peroxidation markers when they are at high levels, are significantly correlated with CVD in subjects with MetS²⁰.

5.6 Conclusions

These data suggest that in these men and women with MetS, measuring both HDL-c concentrations and HDL particles can provide important information about levels and functionality of the protective HDL. In regards to plasma HDL-c, subjects with Low HDL-c appear to have higher risk for biomarkers associated with CVD such as, high TG levels, systolic BP, and WC and lower antioxidant capacity than subjects with Normal HDL-c. We measured lipoproteins size and number and we found that large and medium VLDL and small LDL particles were higher in Low HDL-c group compared with Normal HDL-c group. In contrast, large LDL, which is the one known to be less atherogenic compared to small LDL, was significantly higher in Normal HDL-c group. For that, it appears that in the MetS condition, having normal HDL-c concentrations, the most common atherogenic sizes of lipoproteins are lower compared to subjects with Low HDL-c. Apo A-I was positively correlated with total HDL and large HDL particles. In addition, we were able to measure HDL functionality through measuring PON-1. PON-1 was positively correlated with total HDL but there was no correlation found between PON-1 and large HDL particles, that might due to the fact that we did not have enough subjects to find significance.

5.7 Limitations and Future Directions

In the current study, we were able to measure both HDL-c and HDL particles in 40 subjects with MetS. Measuring both of these parameters can provide more information about the importance and possible relationships with increased CVD risk factors. There were 11 men and 29 women in this study. For that, one possible limitation can be the unequal numbers of each gender, which limits further analysis to be done based on gender type. Another limitation could be the overall number of subjects in each group based on HDL-c concentrations, and this might be one possible reason that many markers were not statistically significant. We were able to measure inflammatory markers such as, TNF- α , CRP, and IL-6 that are used to determine the inflammatory

levels as well as T2D and CVD risk factors. However, we did not measure leptin, which is an adipokine that is involved in the regulation of satiety and energy intake⁴. Development of obesity can cause increased levels of plasma leptin. Leptin is positively correlated with adiposity that can cause leptin resistance, which is common in obese and MetS individuals⁴. Also, elevated plasma leptin levels not only affect satiety and metabolism but also have an effect on BP and increase the peripheral vascular resistance, for hyperleptinemia, which is considered an independent CVD risk factor⁴. Another important marker that we did not measure is adiponectin. Adiponectin is negatively associated with WC, TG, fasting insulin, and blood pressure. Further, adiponectin is positively associated with HDL in which it can enhance PON1 production⁴. Thus, it will be interesting to look to these markers and to check different possible correlations in subjects with MetS. Also, adiponectin expression is reduced by the increase of TNF- α . Since individuals with MetS have increased inflammatory markers and possibly high TG, BP and low HDL-c concentrations, measuring adiponectin will provide interesting information in this high-risk group. Another important point as a future direction is to assess expression for oxidative stress and inflammation related genes in Normal HDL-c groups and Low HDL-c group. One of the limitations of the fluorometric kit used for PON-1 measurement is requires EDTA containing tube. Thus, we faced a technical issue during this measurement. This is one limitation that can be a considered for future studies. It would be useful to utilize another kit such as ELISA and/or using serum PON-1 to measure all PON-1 isoforms to better understand HDL functionality. Another future direction is to look at gender and ethnicity as it relates to MetS, HDL-c, and HDL particles in the data analysis. It has been indicated that gender and certain ethnicities can be different in regards MetS parameters and HDL functionality. Therefore, it will be interesting to examine these factors and how they are different between groups.

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